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Influence of polyploidy on morphology, genetic differentiation and reproductive strategy amongst varieties of *Rhodohypoxis baurii* (Hypoxidaceae).

by
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Submitted in fulfilment of the requirements for the
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in
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in the Faculty of Science, University of the Witwatersrand, Johannesburg,
South Africa

Supervisor: Dr. Kelsey L. Glennon
Co- supervisor: Prof. Glynis V. Goodman-Cron

03 May 2023

Declaration

I declare that this dissertation is my own, unaided work. It has been submitted in fulfilment of the requirements of a Master of Science at the University of the Witwatersrand. It has not been submitted before for any degree or examination to another university or similar institution.



Bianca Tasha Ferreira

3rd May 2023

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Dr. Kelsey Glennon

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TABLE OF CONTENTS

Declaration **2**
TABLE OF CONTENTS **3**
LIST OF TABLES AND FIGURES **4**
ABSTRACT **6**
ACKNOWLEDGMENTS **8**
CHAPTER 1: INTRODUCTION **9**
1.1 Rationale 9
1.2 Literature Review 11
Polyploidy overview *11*
Evolutionary importance of polyploidy *14*
Reproductive isolation and shifts in breeding systems *16*
Species, subspecies and varieties *20*
Rhodohypoxis baurii complex *21*
1.3 Aim and objectives 26
CHAPTER 2: METHODS **27**
Morphometrics Analysis *27*
Cross-pollination viability 39
Agamospermy/apomixis 40
Pollinator identification 40
Pollen viability 41
CHAPTER 3: RESULTS **42**
Morphometric analysis of R. baurii varieties and ploidy levels *42*
Microsatellite analyses - Genetic differentiation and gene flow *53*
Reproductive strategies *58*
Pollinator observations across R. baurii varieties *65*
CHAPTER 4: DISCUSSION **71**
How does polyploidy influence morphology in R. baurii varieties? *71*
Reproductive strategies and escaping MCE in R. baurii *74*
Genetic diversity and gene flow across R. baurii varieties *77*
Latitudinal polyploid gradient *80*
Taxonomic implications *82*
CHAPTER 5: CONCLUSION **87**
REFERENCE LIST **88**
APPENDIX **103**

LIST OF TABLES AND FIGURES

Figure 1: Genetic differentiation, morphological change and changes in polyploidy, when exhibited together, could lead to polyploid speciation events. ……..……..11

Figure 2: The four main regions of the Drakensberg (Northern Drakensberg, Central Drakensberg, Southern Drakensberg and Eastern Cape Drakensberg). ……..22

Figure 3: Images of the three *Rhodohypoxis baurii* varieties……....24

Table 1: Character list for multivariate analysis of *Rhodohypoxis baurii* complex……..……....28

Figure 4: Trichome branching patterns and sheaths across *R. baurii* varieties…….....29

Figure 5: Reproductive morphology measurements in *R. baurii* varieties……...….30

Figure 6: Geographic distribution of *Rhodohypoxis baurii* herbarium specimens used for morphometric analyses. ……..…..32

Table 2: Sampled localities and GPS co-ordinates of 12 populations of *Rhodohypoxis baurii* varieties used in molecular microsatellite analyses and seven populations used in morphometric analyses. ...……..…..33

Figure 7: Distribution map of *Rhodohypoxis baurii* varieties known range, and the 11 sampled populations. ……..34

Figure 8: Inter-varietal and intra-varietal crosses conducted between the three *Rhodohypoxis baurii* varieties with all varieties acting as both pollen donors (paternal) and pollen receivers (maternal). ……...…..40

Figure 9: *Rhodohypoxis baurii* pollen grains stained with Alexander's stain showing viable pollen (red) and inviable pollen (green/blue). ……..……..…..42

Table 3: List of contributions (loadings) of each morphological character measured for the first four Principal Component Axes, based on the PCA analysis……..…..43

Figure 10: Non-metric multidimensional scaling (NMDS) analysis of 116 herbarium specimens of *R. baurii* varieties. ……..44

Figure 11: Comparison of vegetative, and floral morphological traits of 116 herbarium specimens represented by *Rhodohypoxis baurii* varieties. ……..46

Figure 12: Comparison of floral morphological traits of 31 collected greenhouse specimens represented by *Rhodohypoxis baurii* varieties. ……48

Figure 13: Comparison of vegetative morphological traits of 43 herbarium specimens represented by *Rhodohypoxis baurii* varieties. ……..…49

Figure 14: Image showing the size difference of reproductive organs such as anther and ovules between *Rhodohypoxis baurii* varieties. ……..49

Figure 15: Variation in flower colour of *R. baurii* individuals housed in the greenhouse. ……..…...50

Figure 16: Comparison of floral morphological traits across 31 collected greenhouse specimens of *Rhodohypoxis baurii* varieties grouped by ploidy level ……..52

Figure 17: Comparison of vegetative morphological traits of 31 collected greenhouse specimens of *Rhodohypoxis baurii* varieties grouped by ploidy level …….....53

Table 4: Indices of genetic diversity based on 12 microsatellite markers across 11sampled population, the three *Rhodohypoxis baurii* varieties and three ploidy levels. ……...55

Figure 18: Population genetic structure, allelic diversity and ploidy of the *R. baurii* populations based on auto-polyploidized data from 12 microsatellite markers. ……..…57

Figure 19: DAPC based on Bruvo genetic distance matrix grouped by A) taxonomic varieties, B) grouped by ploidy levels, C) grouped by populations. …….58

Table 5: Differences in seed set between inter-varietal and intra-varietal crosses as well as between like ploidy and unlike ploidy crosses. ……60

Table 6: Differences in germination success between seeds from intra-varietal and inter-varietal crosses as well as between like ploidy and unlike ploidy crosses. ……..62

Figure 20: Comparison of pollen viability between (A) *Rhodohypoxis baurii* varieties and (B) different ploidy levels. ……..……...63

Figure 21: Desktop Scanning Electron Microscope (SEM) images of *R. baurii* pollen grains. ……64

Table 7: Differences in clonal reproduction between *Rhodohypoxis baurii* varieties and ploidy levels. ……65

Table 8: Summary of insect activity of species observed on inflorescences of *Rhodohypoxis baurii* at seven sites throughout the Drakensberg during the November 2021– January 2022 flowering season. ……..……..67–68

Figure 22: Insect visitors of *Rhodohypoxis baurii* flowers and evidence of herbivory on the tepals and anthers of *Rhodohypoxis baurii* varieties. ……....69

Figure 23: Frequent insect visitors caught whilst visiting *Rhodohypoxis baurii* flowers at two Northern Drakensberg populations. . ……..…...70

ABSTRACT

Evolutionary mechanisms, such polyploidy (increase in chromosome sets), alters plant morphology, gene flow and reproductive strategies, which can facilitate the generation or loss of species. *Rhodohypoxis L.* (Hypoxidaceae) is a small near-endemic Drakensberg genus comprising six species, one of which is *Rhodohypoxis baurii*. *Rhodohypoxis baurii* contains three morphologically distinct varieties, with varying ploidy-levels: *R. baurii* var. *baurii* (2×, 4×), *R. baurii* var. *platypetala* (2× 3× , 4×), and *R. baurii* var. *confecta* (2×). Therefore, *R. baurii* is an ideal system to evaluate whether polyploidy leads to lineage divergence or homogenization and contributes to biodiversity in this lineage. The aim of the study was to assess the influence of ploidy on morphology, genetic differentiation and reproductive strategy among varieties of *Rhodohypoxis baurii* (Hypoxidaceae), as well as to better recognise the three varieties. Thirty vegetative, floral, and reproductive traits were measured across 124 herbarium specimens and 43 individuals housed in the greenhouse. A matrix containing 20 quantitative and 12 qualitative characters was constructed and a Principal Coordinates Analysis (PCoA) and Non-Metric Multidimensional Scaling analysis (NMDS) were conducted. Important distinguishing morphological features that had a high eigenvalue (shown via a PCA) were selected for direct comparison using box and whisker box plots to compare means ± standard errors (SE). Certain morphological traits such as anther length, peduncle length and tepal sizes differed significantly among the varieties and ploidy levels, with polyploid individuals exhibiting the gigas effect. This was especially evident in *R. baurii* var. *platypetala*, which contained many polyploid individuals and exhibited larger flowers (longer and wider tepals) and larger anthers compared to other varieties.

Rhodohypoxis baurii var. *confecta* and *R. baurii* var. *platypetala* are genetically, geographically and morphologically similar, differing only in flower colour, flower size and peduncle length. However, most of these differences can be attributed to differences in ploidy and /or altitude with *R. baurii* var. *confecta* occurring at higher altitudes and *R. baurii* var. *platypetala* containing multiple ploidy-levels. It is therefore evident that ecological differences and polyploidy have shaped the morphological differences in these two taxa. In addition, *R. baurii* var. *baurii* populations in the

Eastern Cape Drakensberg were morphologically, geographically and genetically distinct from all other populations and varieties, and may be a new/ undescribed taxon; however, this warrants further investigation. Out of 231 experimental crosses, 113 intra-varietal and inter-varietal crosses produced seeds. *Rhodohypoxis baurii* polyploid individuals show a shift away from sexual reproduction to asexual reproduction as they all showed higher rates of clonal reproduction than the diploid individuals. Moreover, crosses between polyploids yielded lower seed sets and lower germination rates than diploid-diploid crosses. Genetic differentiation and gene flow were quantified for 280 individuals among the varieties and ploidy-levels across 11 populations using 12 microsatellite markers labelled with the FAM NED dyes. Leaf material was collected from 237 individuals of *Rhodohypoxis baurii* (*R. baurii* var. *confecta* n = 88, *R. baurii* var. *baurii* n = *87*, *R. baurii* var. *platypetala* n = 62) and flow cytometry conducted to estimate ploidy. A latitudinal ploidy gradient was evident across sampled populations that corresponds with shifts in reproductive strategy, and changes in the extent of gene flow. Population genetic structure coincided primarily with geographic localities, with diploid Northern Drakensberg populations having similar allelic diversity to one another. The Central and Southern Drakensberg mixed ploidy populations also showed similar allelic diversity but differed from the tetraploid Eastern Cape Drakensberg populations. Furthermore, gene flow was higher between geographically close populations irrespective of ploidy-level, with geographically isolated regions (such as the Eastern Cape Drakensberg) and outlying populations (i.e. Karkloof) showing unique genotypes, indicating little gene flow and allele sharing. Consequently, shifts in reproductive strategy and geographic isolation are likely changing gene flow patterns among varieties and ploidy levels which appears to be facilitating both lineage diversification and homogenization in this species.

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CHAPTER 1: INTRODUCTION

1.1 Rationale

Understanding the evolutionary path of mountain endemic plant species helps to quantify, understand the history of, and predict the future of the biodiversity in hotspots such as the Drakensberg Mountain Centre, under the threat of global change. Evolutionary mechanisms, such as whole genome duplication (polyploidy), have been noted to provide advantages in extreme environmental conditions and are often cited for their potential role in ameliorating the harm of global change to biodiversity and ecosystem functionality (Ahrens *et al.*, 2020; Innes *et al.*, 2021; Van de Peer *et al.*, 2017). Polyploidy often results in morphological and reproductive changes which may, subsequently, lead to diversification of plant lineages (Soltis *et al.*, 2014), resulting in an increase in biodiversity. Two opposing views exist on the effects of polyploidization and genome changes within a long-term evolutionary setting. One view is that polyploidization can lead to long term plant diversification and, therefore, is a driver of speciation and biodiversity (De Storme & Mason, 2014; Levin, 1983; Wood *et al.*, 2009). On the other hand, others hypothesize that polyploidization is 'evolutionary noise' or an 'evolutionary dead-end' due to lower diversification rates of polyploids and lower longterm survival, and therefore insignificant to the main process of evolution (Mayrose et al., 2011; Stebbins, 1950; Wagner, 1970) .

Rhodohypoxis baurii (Baker) Nel is an ideal system to evaluate whether polyploidy can lead to speciation and assist in maintaining and creating biodiversity in this group. *Rhodohypoxis baurii* contains three varieties, comprising a range of different ploidy levels: *R. baurii* var. *baurii* (Baker) Nel (2×, 4×), *R. baurii* var. *platypetala* (Baker) Nel (2×, 3×, 4×, and *R. baurii* var. *confecta* Hilliard & B.L. Burtt (2×) (Saito 1975, Glennon unpublished data). Gene flow between varieties and closely related species may be facilitated by polyploidy and reflected in morphology, suggesting that few reproductive barriers exist between *Rhodohypoxis baurii* varieties. Consequently, many shared morphological traits exist between the *R. baurii* varieties, and distinguishing features need to be identified to enable better recognition of these three varieties.

Chromosome duplication has been noted to alter plant morphological traits, for example, resulting in features such as increased flower number per inflorescence and larger reproductive organs (Balao *et al.*, 2011; Knight & Beaulieu, 2008; Robertson *et al.*, 2011). Observed increases in reproductive organ size are indicative of reduced self-incompatibility (Robertson *et al.*, 2011), often resulting in increased occurrences of selfing in polyploid individuals (Barringer, 2008; Robertson *et al*., 2011). However, the reproductive consequences of polyploidy are not fully understood (Vamosi *et al.*, 2007). Therefore, corroborating ploidy-levels with morphological trends in *R. baurii* will further our understanding of the effects that polyploidy may have on plant morphology, reproduction, and its role in lineage diversification. Recent greenhouse observations suggest that *R. baurii* may reproduce clonally or be self-compatible (B. Ferreira, pers. obs.). In this study, individuals of different ploidy and different taxa will be assessed to describe their reproductive biology. Quantifying gene flow and reproductive strategies, together with examining ploidy-level variation in *R. baurii*, will facilitate our understanding of the role that polyploidy may have played in this group's evolutionary past and potentially have in its future trajectory.

The overall aim of the study is to assess the influence of ploidy on differences in morphology and reproductive strategy among varieties of *Rhodohypoxis baurii* (Hypoxidaceae). Using microsatellites, genetic differentiation and gene flow were quantified among the varieties and individuals with different ploidies. Reproductive strategies of each variety were examined in the greenhouse and pollen and seed viability of intra-variety and inter-variety crosses were examined as well as seed viability of selfed individuals. Field-based pollinator observations were conducted to understand what, if anything, is pollinating *R. baurii*. Quantifying reproductive strategies, together with examining ploidy-level and morphological variation, of *R. baurii* varieties, advance our understanding of the species evolutionary past and assist in predicting its trajectory. This information will contribute to our understanding of the role that polyploidy may play in the generation and perpetuation of species (Figure 1).

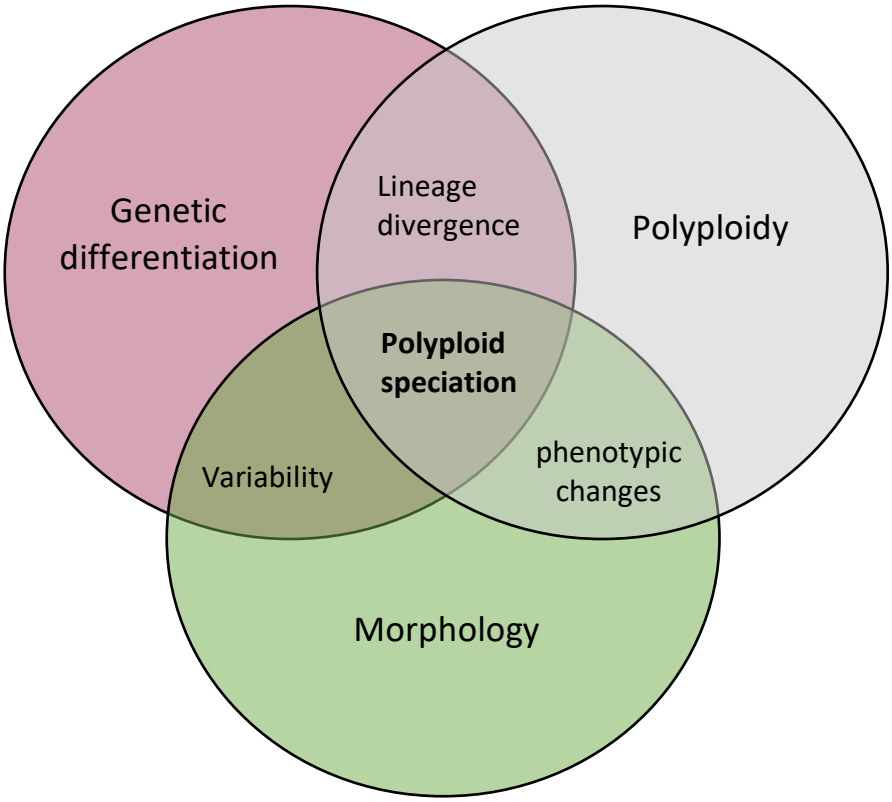

Figure 1. Genetic differentiation, morphological change and changes in polyploidy, when exhibited together, could lead to polyploid speciation events through an increase in variability (both genetic and morphological), phenotypic changes (such as the gigas effect) and lineage divergence.

1.2 Literature Review

Polyploidy overview

Understanding the evolutionary path of polyploid species and the processes involved in speciation events enables one to understand the history and more accurately predict the future of biodiversity. Most speciation theories are based around natural selection, with emphasis on driving selective pressures that cause species divergence and speciation (Mayr, 1963; Schluter & Nagel, 1995; Lynch & Force, 2000; Werth & Windham, 1991). On the other hand, others view selection as any cause of genetic divergence between allopatric populations, resulting in reproductive isolation and subsequent divergence (Sobel *et al.*, 2010). How polyploidy fits into speciation is still an open question (Schluter & Nagel, 1995).

Consequently polyploid events are a likely mechanism of plant diversification and speciation (Sobel *et al.*, 2010; Soltis & Soltis, 2009; Levin & Soltis, 2018; Mayrose *et al.,* 2014); these events result in genetic and phenotypic variation on which natural selection may act.

Polyploid events result in organisms that can be classified into one of two main groups: autopolyploids or allopolyploids. Autopolyploidy is the formation of polyploids within a single species (Clausen *et al.*, 1945; Stebbins, 1947) where multiple sets of chromosomes come from the same taxon (intra-specific hybridisation), whereas allopolyploids result from interspecific hybridisation (Lewis, 1980) and chromosome sets are combined from two different taxa. However, additional types of polyploids were later identified, such as aneuploidy, where chromosome numbers differ from the exact multiple of the base chromosome number (Ramsey & Schemske, 2002) and neopolyploids which are newly formed auto- or allopolyploids (Ramsey & Schemske, 2002). Within each of these broader classifications, polyploid individuals are further identified based on the number of chromosome sets they possess. For example, triploids (3×) contain three full sets of chromosomes and tetraploids (4×) contain four chromosome sets (Ramsey & Schemske, 1998). These polyploids can arise from multiple different, recurrent and independent origins (Soltis *et al.*, 1989; Werth *et al.*, 1985; Wyatt *et al.*, 1988).

Types of polyploid formation

Polyploids can have multiple origins with different mechanisms involved in their formation. Recurrent formation of polyploids from genetically different parents is common and relatively frequent for both allopolyploids (Bayer & Crawford, 1986; Ogihara & Tsunewaki, 1988; Ranker *et al.*, 1989; Werth *et al.*, 1985; Wyatt *et al.*, 1988) and autopolyploids (Shore, 1991; Soltis & Soltis, 1993; Soltis *et al.*, 1989; Wolf *et al.*, 1990). Numerous ancient polyploidization events have occurred across angiosperms (Barker *et al.*, 2009; Blanc & Wolfe, 2004; Bowers *et al.*, 2003; Cui *et al.*, 2006; Jaillon *et al.*, 2007; Paterson *et al.*, 2004; Shi *et al.*, 2010). For example, Soltis, Soltis and Ness (1989) suggested that alumroot, *Heuchera micrantha* Douglas ex Lindl. (Saxifragaceae), had at least three separate origins of autopolyploid formation in the species' evolutionary history based on ancestral chloroplast genomes shared by diploid and tetraploid populations. Due to the multiple origins of

polyploids, polyploid species can assimilate genetic diversity from many sources /populations of diploids (Symonds *et al.*, 2010). This can maintain high levels of 'segregating genetic variation' (Soltis *et al.*, 2014; Soltis & Soltis, 1999; Soltis & Soltis, 2000). From somatic doubling to the generation and importance of unreduced gametes, triploid and tetraploid individuals can be formed via multiple routes (D'Amato, 1952; Khoshoo, 1959; Navashin, 1925; Thompson & Lumaret, 1992). The nonreduction of gametes is one of the main processes responsible for polyploid formation (Brownfield & Köhler, 2011; Ramsey & Schemske, 1998). Unreduced gametes (diploid or 2n gametes) occur via meiotic defects (e.g., nondisjunction) where ploidy-levels are not reduced during meiosis (Bretagnolle & Thompson, 1995; Harlan & deWet, 1975). Similarly, somatic doubling occurs during an error in mitosis where cells are produced with twice the number of normal chromosomes (Bretagnolle & Thompson, 1995; Brownfield & Köhler, 2011). Both the nonreduction of gametes (also known as "meiotic nuclear restitution") and somatic doubling results in 2n gametes which contain full somatic chromosome numbers (Ramsey & Schemske, 1998). The combination of a reduced and unreduced gamete leads to triploid formation and is more common in diploid populations than polyploid populations (Bretagnolle & Lumaret, 1995; Khoshoo, 1959). Likewise, the union of two unreduced gametes is likely to result in tetraploid formation (Ramsey & Schemske, 1998). Selfing via triploids and tetraploids will commonly result in triploid and tetraploid offspring, respectively (Ramsey & Schemske, 1998). Autotriploid gametes are often not functional due to aneuploidy but can mediate and lead to tetraploid production via self-fertilisation or backcrossing with diploids, where triploids act as triploid bridges (Dermen, 1931; Husband, 2000; Johnsson, 1940; Levin, 1975; Ramsey & Schemske, 1998; Yamauchi *et al.*, 2004). These resulting tetraploids generally have functional gametes and can reproduce further (Yamauchi *et al.*, 2004). Self-fertilisation and backcrossing of hybrid triploids with diploids often leads to allotetraploid formation (Ramsey & Schemske, 1998). Polyploid formation can be either maladaptive or advantageous to individual plants and populations. Chromosome doubling may provide a basis for evolution within a population level framework and can either result in individuals that may be adapted to changing environments or may be deleterious to individuals in environments where diploids are well adapted (Levin, 1983).

Evolutionary importance of polyploidy

Due to the dynamic nature of polyploid genomes, there is extensive potential to generate novel genetic variation (Doyle *et al.*, 2008; Flagel & Wendel, 2009; Gaeta *et al.*, 2007; Leitch & Leitch, 2008; Otto & Whitton, 2000), which in turn, may lead to plant speciation or diversification (De Storme & Mason, 2014; Levin, 1983). However, polyploidy is not always viewed as a contributing mechanism of diversification. For instance, Mayrose *et al.*, (2011) described polyploids as "evolutionary dead-ends," arguing that polyploidy does not contribute significantly to evolution. Initially, Stebbins, (1950) and Wagner, (1970) argued that polyploids are more likely to go extinct than diploids and, therefore, are of less evolutionary importance. Even though it is widely understood that polyploids are likely to go extinct expeditiously at the population level (Levin, 1975; Ramsey & Schemske, 1998; Soltis *et al.*, 2010), they are still of evolutionary importance. Polyploids have the potential for rapid adaptation due to their unique genomic background (Otto & Whitton, 2000; Schoenfelder & Fox, 2015; te Beest *et al.*, 2012). Polyploidy has short-term adaptive abilities and the unique characteristic of duplicated genes, and the retention of multiple gene sets, may explain long- term evolutionary transformation (Otto & Whitton, 2000; Van de Peer *et al.*, 2017). Short- term success of polyploids may be due to genetic changes and subsequent increases in genetic variation (Doyle *et al.*, 2008; Parisod *et al.*, 2010; Schoenfelder & Fox, 2015; Soltis *et al.*, 2014 ;Otto & Whitton, 2000; Van de Peer *et al.*, 2017).

The increase in genetic variation due to polyploidy *per se* affects the morphology and ecology of neopolyploids and may alter interspecific interactions and gene flow (McCarthy *et al.*, 2016; Soltis *et al.*, 2014; te Beest *et al.*, 2012). Furthermore, duplicated genes can have new or different and diverse functions leading to adaptive advantages in polyploids (Adams & Wendel, 2005; Lynch & Force, 2000; Madlung, 2013; Soltis & Soltis, 1993). Polyploids have also played a historically advantageous role in angiosperm diversification (WeissSchneeweiss *et al.*, 2013). Because polyploids often arise from multiple origins, there can be an increase in genomic diversity within polyploid individuals and populations (Soltis & Soltis, 1999; Weiss-Schneeweiss *et al.*, 2012). It has also been documented that there is a positive correlation between species richness and frequency of polyploidy as genera with 50–

75% polyploid species also show greater species richness (Otto & Whitton, 2000; Petit & Thompson, 1999; Vamosi & Dickinson, 2006). Likewise, it has been noted that species richness increases drastically in many angiosperm clades after ancient whole genome duplication events, which supports the suggestion of higher diversification rates in polyploids (Soltis *et al.*, 2009). Furthermore, Crow & Wagner, (2006) found reduced risk of extinction and increased rates of evolution in polyploids. Therefore, despite arguments to the contrary there is compelling evidence that polyploidy may lead to plant speciation and diversification. However, for polyploidy to contribute to evolutionary processes, polyploid individuals first need to form and, more importantly, establish for the effects of polyploidy (be it advantageous or detrimental) to have a long-term impact.

Two stages are crucial to polyploid evolution and success: formation and establishment. These two early stages of polyploid evolution entail that the new cytotype must be viable and fertile in order for the organism and lineage to have a higher likelihood of persisting (Ramsey & Schemske, 2002). The likelihood of new polyploid establishment and survival depends on the extent of reproductive isolation (RI) within and between ploidy levels (Ramsey & Schemske, 1998). Genetic incompatibility and reproductive isolation (RI) are often a consequence of polyploidization. Polyploidization can have detrimental effects on plant fertility due to the unstable nature of polyploid genomes and the meiotic aberrations that exist (Van de Peer *et al.*, 2017). Yet, the main hindrance to the success of newly formed polyploid individuals is minority cytotype exclusion (MCE) (Levin, 1975; Van de Peer *et al.*, 2017). MCE is where one cytotype is dominant (often diploids) and more common than other or new cytotypes (such as triploids or tetraploids). The less common cytotypes are less likely to mate and reproduce due to a lack of like ploidy partners (Husband, 2000). The reproductive success of a new cytotype is therefore frequency dependent as the minority cytotypes are at a mating disadvantage (Levin, 1975). If minority cytotypes do not overcome MCE, they will be rapidly excluded and eventually go extinct in the population (Levin, 1975). In order to overcome MCE, polyploid individuals need to have like-polyploid mates in a diploid dominated environment in order to reproduce (Van de Peer *et al.*, 2017) or must be able to self-pollinate. Polyploidization often occurs with a shift in breeding systems from cross-

pollination or sexual reproduction to self-pollination or asexual reproduction respectively (Comai, 2005).

Reproductive isolation and shifts in breeding systems

Selfing in polyploid individuals is aided by a breakdown in self-incompatibility (Levin, 1983). A decrease in self-incompatibility allows self-pollination to occur, leading to further polyploid formation (Ramsey & Schemske, 1998) and an escape from MCE. This change in reproductive systems due to chromosome doubling is relatively common in many taxa, such as *Solanum* L.*, Trifolium repens* L.*, T. hybridium* L. and *Lycopersicon peruvianum* L. (Brewbaker, 1954, 1958; de Nettancourt *et al.*, 1974; Lewis, 1943; Magoon *et al.*, 1958). Assortative mating by cytotypes may aid in overcoming MCE (Mable *et al.*, 2011), but selfpollination and asexual reproduction tend to be more common than sexual reproduction, particularly in neopolyploids (Alix et al., 2017; Ramsey & Schemske, 2002). New allopolyploids formed via hybridization often show agamospermic/apomictic reproductive strategies (Alix *et al.*, 2017). For example, *Sorbus* L. species are known for producing microspecies via hybridisation that exhibit apomictic reproduction (Robertson *et al.*, 2011). There are clear similarities and shared characteristics between polyploid formation and gametophytic apomixis: both processes rely on the formation of unreduced gametes (Majeský *et al.*, 2012; Ramsey & Schemske, 1998; Whitton *et al.*, 2008) and agamospermy is almost exclusively restricted to polyploids in *Erigeron annuus* (L.) (Noyes & Rieseberg, 2000).

Mating systems can influence and change the long-term evolutionary trajectory and success of polyploids (Barrett *et al.*, 2003; Barrett *et al.*, 1996; Barringer, 2007; Lande & Schemske, 1985; Stebbins, 1950). Many questions exist about the evolution of self-fertilization or asexual reproduction and the trade-offs associated with this shift in breeding system (Barringer, 2007). Self-fertilization can be either advantageous as a “reproductive assurance mechanism” (Morgan & Wilson, 2005; Pannell & Barrett, 1998; Stebbins, 1950) or detrimental as it can result in inbreeding depression (Charlesworth & Charlesworth, 1987; Lande & Schemske, 1985). Even though the pattern of increased self-pollination with changes in ploidy has been noted and accepted widely, the relationship and mechanism between self-fertilization and polyploidy is poorly understood (Barringer, 2007; Mable, 2004; Stebbins, 1950). However, several hypotheses exist around why polyploidy may be associated with increased levels of selfing (Barringer, 2007).

Polyploidy usually results in a breakdown of self-incompatibility, resulting in increased frequency of selfing events (Barringer, 2007; Mable, 2004). On the other hand, the ability to self-fertilize may facilitate polyploid evolution (Barringer, 2007). Neopolyploids are likely to be in sympatry with their diploid progenitors when they first form, and because crosses between different cytotypes often lead to reduced fitness (Jackson, 1976; Levin, 1975; Ramsey & Schemske, 1998), minority cytotypes experience MCE and minority cytotype disadvantages (Levin, 1975). Therefore, the ability to self-pollinate allows polyploid individuals to escape MCE, survive, and reproduce (Levin, 1975; Ramsey & Schemske, 1998; Stebbins, 1971). Lastly, polyploids may exhibit higher levels of self-fertilization as they experience less inbreeding depression than diploids due to the multiple copies of genes they possess (Lande & Schemske, 1985). Increased selfing without the consequences of inbreeding depression would result in the selection of increased selfing instances in polyploid individuals and populations (Barringer, 2007).

Asexual reproduction is another common breeding system shift in many polyploid individuals (Levin, 1983). A shift to asexual reproduction with polyploidization is clearly seen in *Themeda australis* Stapf. as diploids reproduce sexually, yet tetraploids reproduce asexually (Woodland, 1964). In addition to selfing, reproductive isolation (RI) of polyploids and MCE can be facilitated by changes in pollinator interactions between diploid and polyploid individuals due to changes in polyploid morphology (McCarthy *et al.*, 2016; Van de Peer *et al.*, 2017). Increased genetic variation among polyploids may lead to differences and divergence in morphological traits of polyploid individuals (McCarthy *et al.*, 2016; Soltis *et al.*, 2014). Changes in traits that attract pollinators (such as inflorescence colour, size and number of inflorescences per individual) will lead to changes in pollinator interactions (Gross & Schiestl, 2015; McCarthy *et al.*, 2015; Segraves & Thompson, 1999). Consequently, pollinators may avoid or favour polyploids due to their different flower morphology and/or chemical scent/compounds, leading to assortative mating and resulting in RI of polyploids (Porturas *et al.*, 2019; Segraves & Anneberg, 2016).

Changes in floral morphology (and the subsequent change in pollinator relationships) are relatively common in polyploid taxa (Taylor & Smith, 1980). Higher ploidy-levels often exhibit increases or decreases in the number of inflorescences/flowers per

individual plant compared to their diploid progenitors (Levin, 1983). For example, in White clover, *Trifolium repens* L. (Fabaceae) tetraploid individuals showed a 20% decrease in number of flowers per head, whereas in Strawberry clover, *T. fragiferium* L.*,* and Egyptian clover, *T. alexandrinum* L.*,* tetraploids showed a 20% increase in the number of flowers per head (Mackiewicz, 1965). Similarly, Honey clover, *Melilotus albus* Medik. (Fabaceae) tetraploids also showed an increase in flowers per head by 30% (Jaranowski & Kalasa, 1971). Interestingly, RI due to differentiation in pollinator communities (as a result of morphological changes) is not necessarily negative but may promote speciation events (Van de Peer *et al.*, 2017). Differentiation in pollinator communities in a mixed population of diploid and polyploids will often cause RI, which may facilitate polyploid establishment (Segraves & Anneberg, 2016). If the "new" polyploid traits are selected for by pollinators, MCE will be overcome, and establishment success will increase, subsequently, a speciation event is more likely to occur (Van de Peer *et al.*, 2017).

Polyploidization not only leads to changes in floral morphology, but can cause a multitude of morphological changes induced by chromosome duplication (Balao *et al.*, 2011; Stebbins, 1947). After whole genome duplication, polyploids experience processes that can shrink or enlarge their genomes (Leitch & Leitch, 2008). This genome modification can induce phenotypic variation (Bennet & Leitch, 2005; Chen, 2007). Cell enlargement and an increase in trait sizes is a well understood and an accepted effect of whole genome duplication (Knight & Beaulieu, 2008; Levin, 2002; Stebbins, 1971). Known as the 'gigas effect', the size of organs generally increases with chromosome number (Müntzing, 1936; Otto & Whitton, 2000; Stebbins, 1971). The 'gigas effect' is more common in new polyploids (Ramsey & Schemske, 2002). The underlying process behind this relationship is known as the "nucleotype effect" where organs are larger merely because the cells comprising them are larger (Bennett, 1971; Doyle & Coate, 2019). However, the 'gigas effect' is not consistent for all species (Otto & Whitton, 2000; Vamosi *et al.*, 2007) as many polyploid taxa exhibit smaller or equal sized phenotypic traits compared to their diploid progenitors (Ning *et al.*, 2009; Porturas *et al.*, 2019; Segraves & Thompson, 1999; Trojak-Goluch & Skomra, 2013). For example, in Common hop, *Humulus lupulus* L. (Cannabaceae), tetraploid individuals have larger cones (infructescenses) compared to diploid individuals but similar sized flowers and significantly smaller leaves than diploids

(Trojak-Goluch & Skomra, 2013). Phenotypic changes in reproductive traits are commonly seen in polyploids, such as larger pollen grains (Porturas *et al.*, 2019). In some Solanaceae species, for example, polyploids had larger reproductive organs than their diploid progenitors (Robertson *et al.*, 2011). Other common morphological changes in polyploid individuals include increases in stomatal diameters (Mtileni et al., 2021), changes in petal length (Bennett, 1971), changes in plant stature (Balao *et al.*, 2011), thicker foliage and thicker stems (Ramsey & Schemske, 2002). Changes in reproductive morphology due to polyploidization not only lead to changes in reproductive strategies but can also result in changes in phenology. These shifts often affect pollinator interactions and may affect gene flow and genetic diversity within and between populations and species (Ramsey & Schemske, 2002). Due to the larger morphological structures found in polyploids, and the larger cells, developmental processes tend to take longer (Cavalier-Smith, 1978; Ramsey & Ramsey, 2014) as more time is needed for cells to divide (Bennett, 1987; Francis *et al.*, 2008). This can potentially result in later flowering times and a shift in flowering phenology in polyploid individuals (Jersáková *et al.*, 2010; Oswald & Nuismer, 2011; Roccaforte *et al.*, 2015; Segraves & Anneberg, 2016). Delays in, and prolonged flowering times are common in neopolyploids where growth and development are slowed (Ramsey & Schemske, 2002). For example, induced autotetraploids of *Medicago* L. and *Melilotus* Mill.. (Fabaceae) species exhibited flowering times that were several weeks longer than their diploid counter parts (Jaranowski & Kalasa, 1971).

Changes in morphology, reproductive strategies, flowering phenology and the subsequent changes in pollinator interaction, together, may alter gene flow and allele sharing patterns. The breakdown in self incompatibility combined with changes in pollinator interactions leading to changes in gene flow can facilitate hybridisation. Lower reproductive incompatibility and fewer interspecific reproductive barriers often lead to higher levels of gene flow and allele sharing among distinct taxa (Alix *et al.*, 2017; Schluter & Nagel, 1995), which subsequently can result in a higher numbers of hybrid individuals or can facilitate hybrid swarms (Alix *et al.*, 2017; Wood *et al.*, 2009). Hybridization often results in introgression, where recurrent backcrossing creates new allele combinations, generating genetic diversity and possibly driving diversification and speciation (Rieseberg, 1995). Therefore, hybridization may cause high genetic variability by producing new or novel genotypes, potentially leading to

speciation events (Alix *et al.*, 2017). Hybridization together with polyploidization may act as drivers of evolutionary diversification by increasing gene flow, resulting in speciation (Alix *et al.*, 2017; Levin, 2002; Ramsey & Schemske, 1998; Rieseberg, 1995). High levels of gene flow can lead to the homogenization of distinct taxa or can produce new and novel lineages (Ramsey & Schemske, 1998). The resulting homogenization or diversification often blurs species boundaries, resulting in the division or combination of species into subspecies or varieties, depending on the degree of reproductive and geographic isolation.

Species, subspecies and varieties

Historically, species were recognised according to morphological similarities (Mayr, 1969), but are now defined in a multitude of ways based on various species concepts. These species concepts are based on phenotypic distinguishability, reproductive isolation, ecological niche occupation, and lineage monophyly through time (phylogenies) (Coyne & Orr, 2004; de Queiroz & Donoghue, 1988). A species is the primary taxonomic unit and the basic evolutionary unit (Radford, 1986). There are many different and varied definitions of a species with several different concepts and criteria for recognizing species. The unified species concept recognises species as separately evolving metapopulation lineages as the only criterion necessary for describing distinct species. (De Queiroz, 2007). However, this concept does not assist in identifying differences between isolated populations or varieties that may or may not be evolving separately. Therefore, for the purposes of this study, a species is defined as the smallest group of populations which is permanently distinct and distinguishable from another with a reasonable degree of reproductive isolation from other species under natural conditions (following Cronquist, 1968). However, infraspecific classifications (e.g., subspecies, variety) are required when species lines are blurred and there is recognisable variation within a taxon that has a geographic or ecological link (Du Rietz, 1930; Stace, 1989).

Traditionally, subspecies are a taxonomic rank lower than species, and many authors use subspecies and varieties interchangeably as synonyms (Hamilton & Reichard, 1992). Nonetheless, Du Rietz (1930) defined both subspecies and varieties and these definitions are widely accepted and are currently used in botanical classification. Subspecies are defined as a population of several biotypes forming a relatively distinct

regional group (Du Rietz, 1930) and, therefore, are geographically isolated ecotypes or "topodemes" (Stace, 1989). Varieties are defined as one or several populations of one or several biotypes forming a somewhat distinct local group (Du Rietz, 1930) and, therefore, are local or ecological ecotypes or "topodemes" (Stace, 1989).

Species may also be defined by the "taxonomic circle" (Damm *et al.*, 2010). In this framework, organisms or populations can only be defined as a species when at least three types of data, with DNA data as the foundation, support the species' recognition. These additional data sources include reproductive isolation, ecological data such as habitat information, geographical data such as the species distribution, and morphological data (Damm *et al.*, 2010; DeSalle *et al.*, 2005; Fitze *et al.*, 2011). However, this approach cannot be applied at subspecies or varietal levels due to the lack of reproductive isolation and the morphological and ecological differentiation often seen between infraspecific taxa, especially varieties (Clausen, 1941). Populations of subspecies are usually geographically isolated from one another and, therefore cannot interbreed *in situ*, however, they may not be reproductively isolated and when in sympatry such subspecies could potentially share alleles. Similarly, populations of varieties that are close enough to each other (not in sympatry, but not completely isolated) will likely interbreed.

Rhodohypoxis baurii complex

Rhodohypoxis is a small genus within the Hypoxidaceae that comprises six species (Hilliard & Burtt, 1978). *Rhodohypoxis* is a near endemic montane to alpine genus distributed in eastern South Africa, centred in the Drakensberg Mountain Centre and Lesotho plateau with additional populations found in the KwaZulu-Natal Midlands, Free State and Mpumalanga (Hilliard & Burtt, 1978) (Figure 2). *Rhodohypoxis* is distinguished from *Hypoxis* (its sister taxon) by its white and pink flowers (versus yellow), the presence of a short perigone tube and subsessile anthers arising from the perigone at two levels (Hilliard & Burtt, 1978). Other distinguishing features of the genus are trichomes (hirsute/pilose) on the leaves, pedicels, and ovary, and a tufted indumentum on the back of the outer tepals. The leaves are basal and either spreading on the soil surface or erect, elliptic to filiform. The anther thecae have acute, usually

free tips. Stigma lobes consist of three fleshy papillose flanges attached to a very short style. Seeds are either ellipsoid or spherical (Hilliard & Burtt, 1978).

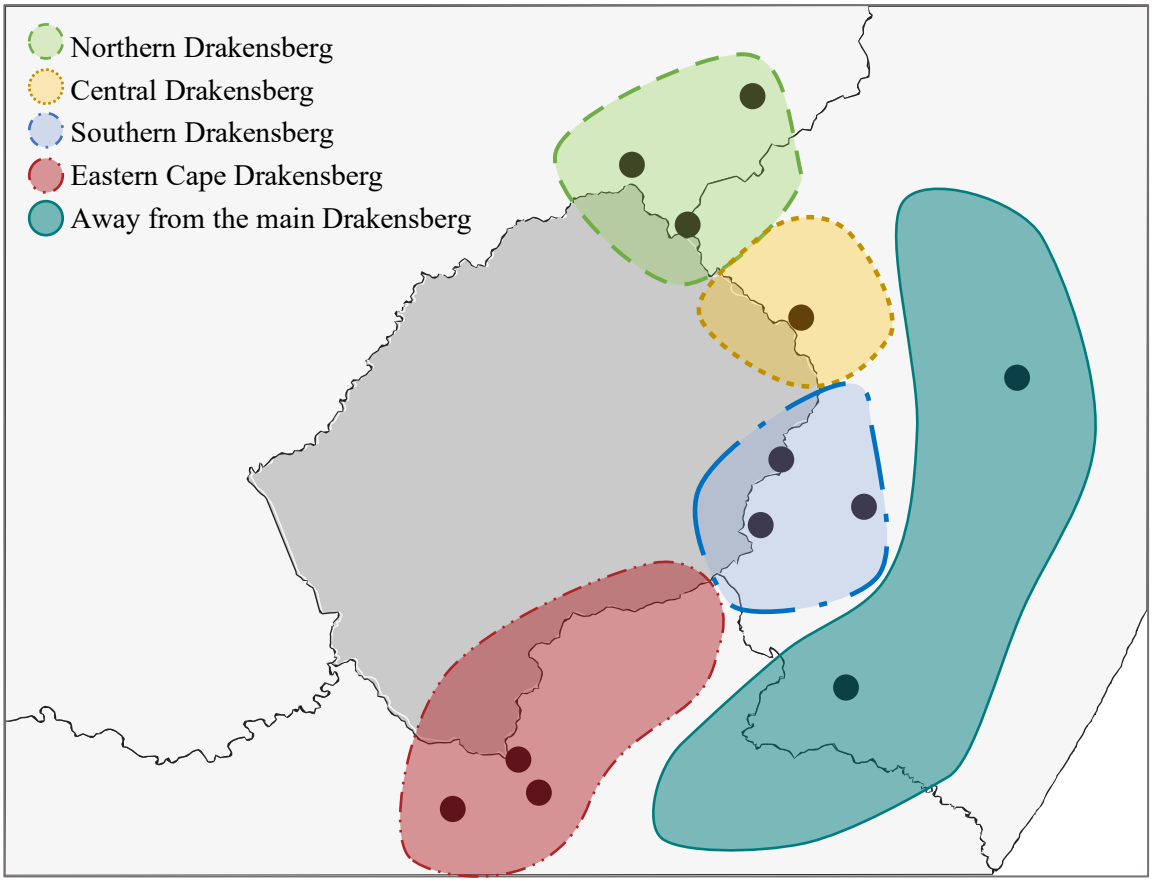

Figure 2. The four main regions of the Drakensberg (Northern Drakensberg, Central Drakensberg, Southern Drakensberg and Eastern Cape Drakensberg) with black points showing the 11 sampled populations, including two populations with localities described as "away from the main Drakensberg" by Hilliard & Burtt (1978).

Rhodohypoxis baurii is the type for the genus and is characterised by Hilliard and Burtt (1978) as having 4–10 leaves that are linear-lanceolate to lanceolate and hairy on the upper surface and margins, anthers ranging from 1.5–2 mm, ovaries 2–4 mm in diameter, and seeds 1.5 mm in diameter on average. *Rhodohypoxis baurii,* commonly known as the 'red star,' is a morphologically variable species containing three varieties: *Rhodohypoxis baurii* var. *baurii* , *R. baurii* var. *platypetala*, and *R. baurii* var. *confecta* (Hilliard & Burtt, 1978) (Fig. 3). *Rhodohypoxis baurii* var. *baurii* has been noted to have two distinct morphological forms associated with geographic distribution (Hilliard & Burtt, 1978). The first form occurs "away from the main Drakensberg", is found at lower altitude (1140 – 1740 m a.s.l.) and has larger

leaves, whilst the other form occurs “on the main Drakensberg” or “facing the main berg at heights” and occurs at higher altitudes (2070 – 2640 m) and has smaller leaves (Hilliard & Burtt, 1978, p. 57). Several taxonomic changes have been made within the species’ history ((Hilliard & Burtt, 1978). Most notably, *R. milloides* (Baker) Nel. was originally considered a variety of *Rhodohypoxis baurii* by Jahrbücher in 1914 *(R. baurii* var. *milloides* (Hilliard & Burtt, 1978). When the genus was revised by Hilliard & Burtt in 1975 it was given species status as *Rhodohypoxis milloides* (Hilliard & Burtt, 1978). The most obvious and variable characters among the three varieties of *R. baurii* are the flower number and colour as flowers are either solitary or paired and can be pink, white, red or a mix within a single population.

R. baurii var. *baurii* and *R. baurii* var. *confecta* both have erect leaves while *R. baurii* var. *platypetala* may have either spreading or erect leaves (Hilliard & Burtt, 1978). Regarding flower colour, *R. baurii* var. *baurii* flowers tend to be red and pink or very rarely white. In contrast, *R. baurii* var. *platypetala* generally has white flowers that are rarely pigmented, and *R. baurii* var. *confecta* flowers tend to be white, red or pink (Hilliard & Burtt, 1978). These three varieties differ mainly in habitat and show slight but definite ecological distinctions (Hilliard & Burtt, 1978). *R. baurii* var. *baurii* is found in moist habitats on damp and partly shaded cliff faces or in other damp habitats such as in small marshes and streambanks. *Rhodohypoxis baurii* var. *confecta* occurs in moist habitats on damp grass slopes, often among rocky outcrops or between rock sheets or on summits of plateaus in short damp turf among rock sheets and often occurs at higher altitudes (Hilliard & Burtt, 1978). In contrast, *R. baurii* var. *platypetala* is found in dry habitats, often on shallow stony soils on rock sheets at lower altitudes (Hilliard & Burtt, 1978). Even in mixed populations that contain multiple varieties, *R. baurii* var. *platypetala* is found on drier ground than the other varieties. *R. baurii* var. *confecta* occurs at high altitudes (i.e., above 2600 meters above sea level (m a.s.l.) and forms sheets of mixed white, pink and red flowers (Hilliard & Burtt, 1978).

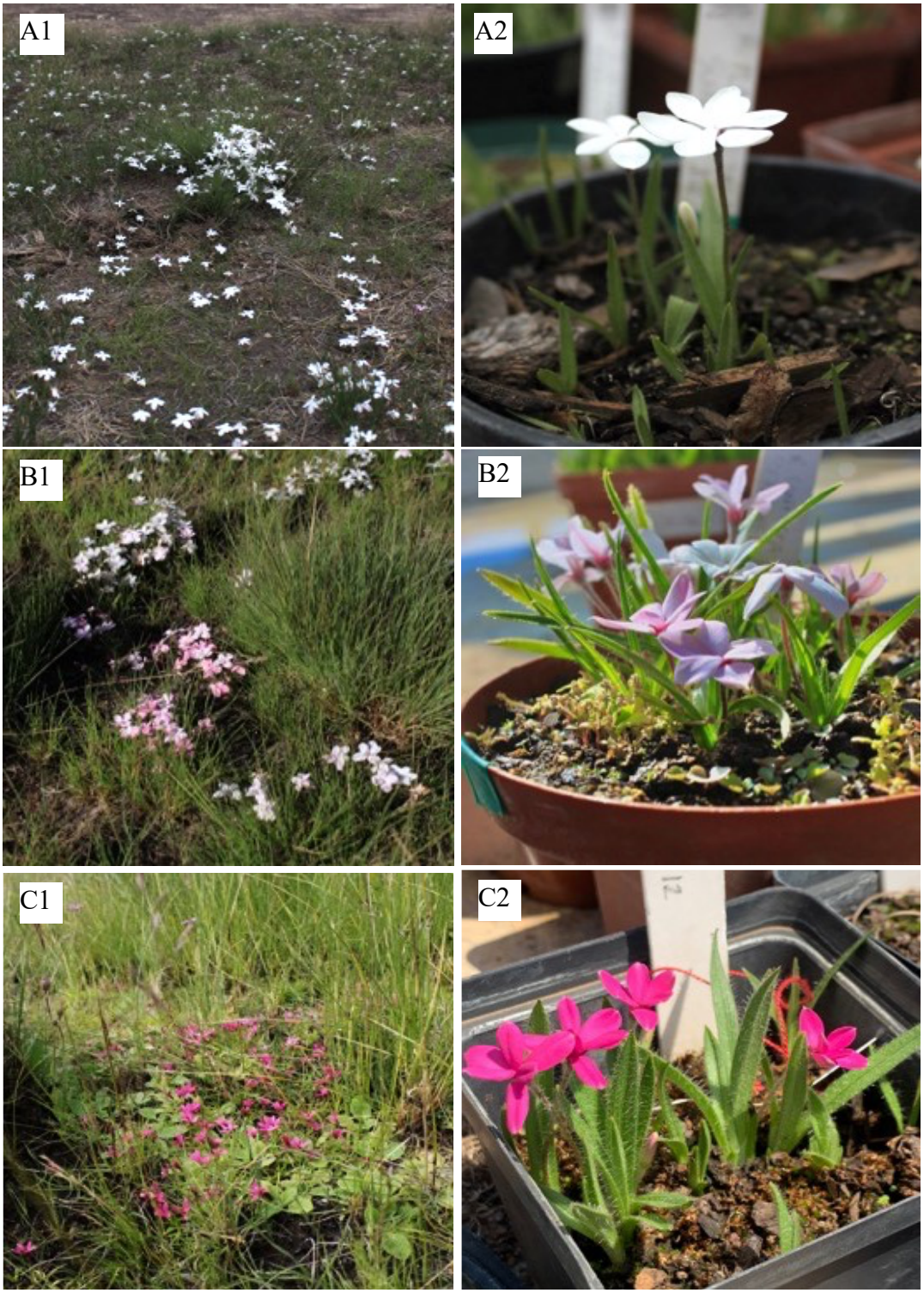

Figure 3. *Rhodohypoxis baurii* varieties A = *Rhodohypoxis baurii* var. *platypetala,* B = *Rhodohypoxis baurii* var. *confecta,* C = *Rhodohypoxis baurii* var. *baurii.* Varieties shown in the field (A1 = Karkloof, B1 = Sentinel, C1 = Tenahead Lodge) and individuals housed in the greenhouse (A2, B2, C2).

Rhodohypoxis populations are morphologically variable and isolated hybrid individuals and populations as well as introgressed populations occur (Hilliard & Burtt, 1978). This suggests that there may be few genetic barriers between varieties. Where *R. baurii* var. *baurii* and *R. baurii* var. *platypetala* occur sympatrically, hybrid lineages form, but are restricted to intermediate habitats and do not lead to merging of parent populations (Hilliard & Burtt, 1978; Glennon pers. obs.). The degree of inter- and intra-specific allele sharing and gene flow that may occur between the species and varieties in *Rhodohypoxis* is unknown. Little is known about the reproductive strategies of *R. baurii*, yet the frequency of hybridization and allele sharing that have been observed suggest that few reproductive barriers exist between *R. baurii* varieties and other *Rhodohypoxis* species. Preliminary cytological analyses indicate varying levels of ploidy in and among the three currently recognised varieties: *R. baurii* var. *baurii* (2×), *R. baurii* var. *platypetala* (2×, 3× 4×), and *R. baurii* var. *confecta* (2×, 3×). Yet, the impacts of these differing ploidy-levels on reproduction and interbreeding are not fully understood. Due to the likely high levels of gene flow between the varieties, distinguishing morphological features are largely lacking. Gene flow between varieties and closely related species may be facilitated by polyploidy and reflected in morphology. Quantifying morphology, potential reproductive isolation, and reproductive strategies, together with assessing ploidy-level variation, in *R. baurii* will be useful to describe the role that polyploidy may have played or potentially will play in this group's evolutionary past and its future trajectory. Therefore, the overall aim of this study is to assess the influence of ploidy on divergence in morphology, genetic differentiation and reproductive strategy amongst varieties of *Rhodohypoxis baurii* (Hypoxidaceae) as well as to better distinguish the three *Rhodohypoxis baurii* varieties.

1.3 Aim and objectives

The overall aim of this study is to assess the relationship between ploidy and variation in morphology, genetic differentiation and reproductive strategy amongst varieties of *Rhodohypoxis baurii* (Hypoxidaceae) as well as to better distinguish the three *Rhodohypoxis baurii* varieties.

Four main objectives are associated with the aim of this study:

1. Use morphometric tools to assess how the three *Rhodohypoxis baurii* varieties may be morphologically distinguished from one another, and identify if morphological differences coincide with ploidy rather than taxonomy.
2. Use molecular data to estimate the gene flow, genetic differentiation and genetic diversity between and among the three *R. baurii* varieties.
3. Evaluate if different varieties or ploidies in *R. baurii* differ in reproductive morphology and/or adopt different reproductive strategies.

CHAPTER 2: METHODS

Morphometrics Analysis

A total of 126 herbarium specimens of *Rhodohypoxis baurii* were examined from Pretoria National Herbarium (PRE), H.G.W.J. Schweickerdt Herbarium (PRU), CE Moss Herbarium (J) and Bews Herbarium (NU) for morphometric analysis (Appendix, Table 1), including the lectotype of *R. baurii* var. *confecta* and specimens from the type localities of *R. baurii* var. *baurii* and *R. baurii* var. *platypetala* that were good matches of the type specimens (as compared with scans received from K and TCD respectively). The specimens were representative of the morphological variation across the geographical distribution of the species and its three varieties (Fig. 6). The specimens were initially sorted into varieties/groups based on the collector's identification, then further sorted based on the distinguishing characteristics noted in the original descriptions by Hilliard & Burtt, (1978). For each specimen, 21–32 floral, vegetative and reproductive characters were measured depending on the quality and completeness of the specimens used (Table 1; Fig. 4; Fig. 5). The locality of each specimen as well as altitudinal data were recorded. Mature leaves and mature flowers were used for all leaf and flower related measurements, respectively. A minimum of three measurements were taken for each feature and the average was log transformed and used for analysis. Qualitative features were scored as either binary or ordered multi-state characters and used to construct a numerical matrix for analysis using PCoA and NMDS. The analyses were conducted on both the quantitative and qualitative traits separately as well as on the combine matrix.

Table 1. Character list for multivariate analysis of *Rhodohypoxis baurii* complex including all floral, vegetative, and reproductive traits. For all trichome branching pattern types (traits 18–21) and sheath types (trait 23), refer to Figure 4 below, and for reproductive traits (traits 26–32) refer to Figure 5 below.

Characters	**Averaging / Coding**			
Peduncle length (mm)	Single measurement			
Leaf lamina length (mm)	Average of 3 measurements			
Leaf lamina width (at widest point)(mm)	Average of 3 measurements			
Receptacle diameter (mm)	Single measurement			
Peduncle trichome length (mm)	Average of 3 measurements			
Receptacle trichome length (mm)	Average of 3 measurements			
Leaf trichome length (mm)	Average of 3 measurements			
Tepal trichome length (mm)	Average of 3 measurements			
Flowers/ peduncle (mm)	??			
Outer tepal length (mm)	Average of 3 measurements			
Outer tepal width (mm)	Average of 3 measurements			
Inner tepal length (mm)	Average of 3 measurements			
Inner tepal width (mm)	Average of 3 measurements			
Trichome density on peduncle	Absent (0)	Sparse (1)	Dense (2)	
Trichome density on leaf lamina	Absent (0)	Sparse (1)	Dense (2)	
Trichome density on receptacle	Absent (0)	Sparse (1)	Dense (2)	
Trichome density on tepal	Absent (0)	Sparse (1)	Dense (2)	
Trichome branching pattern peduncle	Simple (0)	Bifid (1)	Stellate even (2)	Stellate uneven (3)
Trichome branching pattern leaf lamina	Simple (0)	Bifid (1)	Stellate even (2)	Stellate uneven (3)
Trichome branching pattern leaf margin	Simple (0)	Bifid (1)	Stellate even (2)	Stellate uneven (3)
Trichome branching pattern tepal	Simple (0)	Bifid (1)	Stellate even (2)	Stellate uneven (3)
Flower colour	White (0)	Light pink (1)	Dark pink (2)	
Spiky fibrous sheaths	Present (0)	Absent (1)		
Tufted trichomes on tepal tips	Present (0)	Absent (1)		
Trichome density pattern on leaves	Absent (0)	Sparse (1)	Dense (2)	
Anther length	Average of 3 measurements			
Anther width	Average of 3 measurements			
Anther lobe width	Average of 3 measurements			
Stigma length	Single measurement			
Stigma lobe length	Single measurement			
Stigma lobe width	Single measurement			
Ovule diameter across centre	Average of 3 measurements			

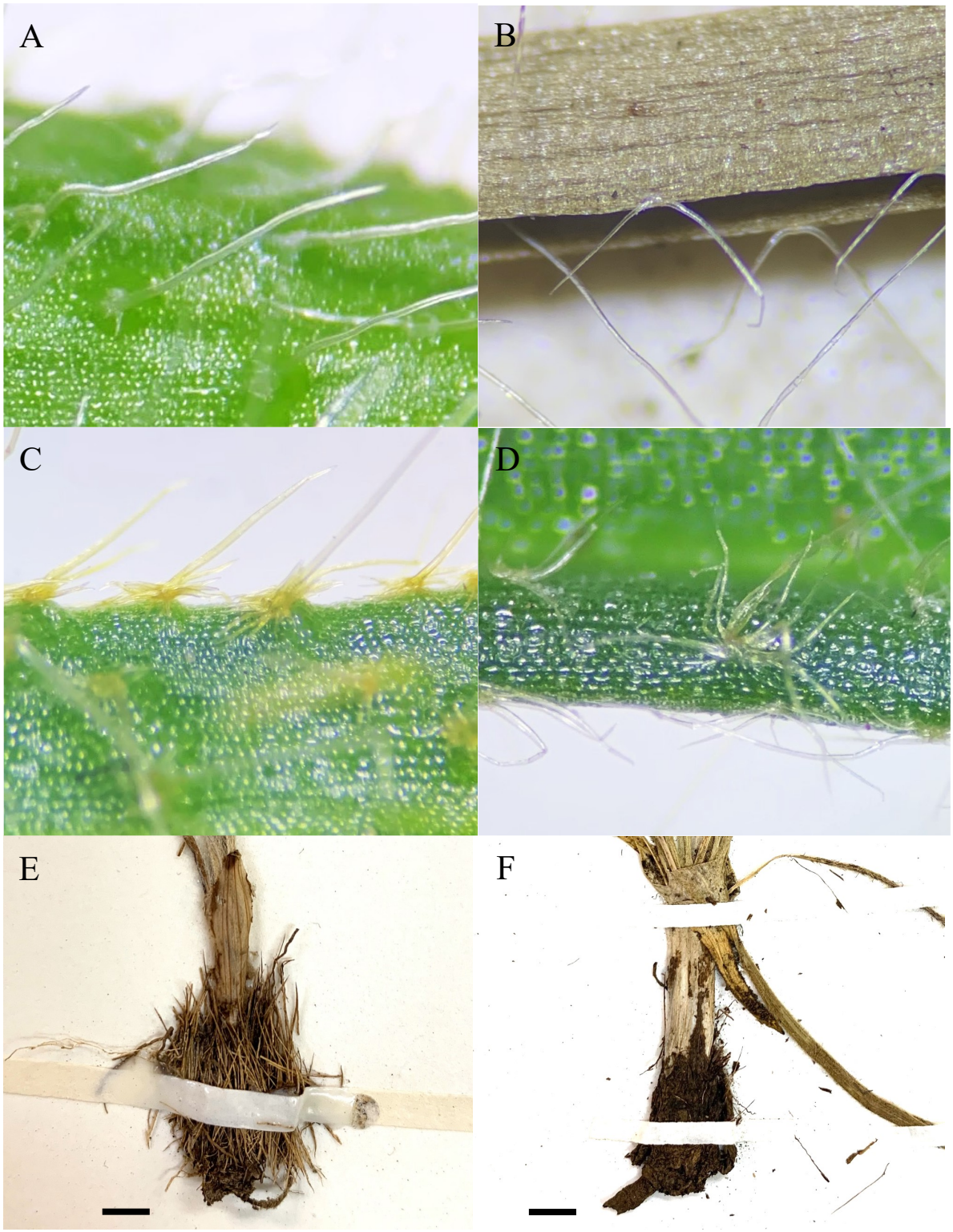

Figure 4: A–D**)** Trichome branching patterns on leaves across *R. baurii* varieties showing (A) single trichome on *R. baurii* var. *baurii* from Hebron Farm, (B) bifid trichome on *R. baurii* var. *baurii* from Impendhle region in Natal, (C) uneven stellate trichome with a single long trichome surrounded by a stellate cluster at the base seen on *R. baurii* var. *confecta*, and (D) typical even stellate trichome on *R. baurii* var. *baurii* from Vrederus. E–F). Differences in sheaths surrounding the base on the peduncle and leaves just above the bulb with (E) showing 'spiky' fibrous sheath hairs on *R. baurii* var. *platypetala* from Karkloof and (F) showing a smooth sheath on *R. baurii* var. *confecta* from Sentinel population. Scale bar = 2.8 mm.

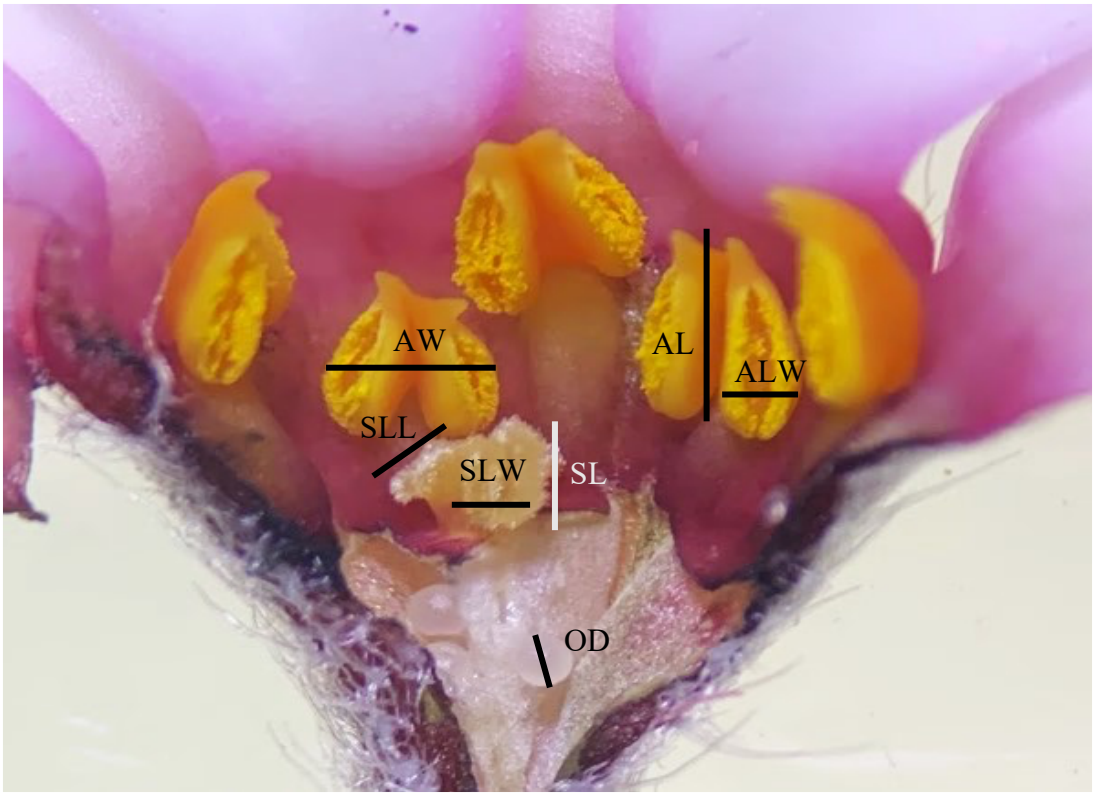

Figure 5. Reproductive morphology measurements in *R. baurii* varieties: (AL) Anther Length, (AW) Anther Width, (ALW) Anther Lobe Width, (SLL) Stigma Lobe Length, (SLW) Stigma Lobe Width, (OD) Ovule Diameter, (SL) Stigma Length**.**

Morphometric analysis of the 32 morphological features of 126 specimens was used to confirm the identity of the three putative *Rhodohypoxis baurii* varieties. A matrix containing 20 quantitative and 12 qualitative characters was constructed using the measurements of the characteristics for all the specimens. A PCA was conducted on the combined data to generate eigenvalues. A PCA was conducted on both logged transformed measurements and unlogged measurements. Both logged and unlogged data resulted in the similar values and trends were the same between logged and unlogged data. Traits that exhibited very low eigenvalues (<0.0001), indicating a lack of variability, were removed from the data set (Appendix, Table 3). Traits that were not significant and did not help distinguish the three varieties were removed. A total of 8 traits were removed (tufted trichomes on tepal tips, peduncle trichome length, tepal trichome length, trichome density on peduncle, trichome branching pattern peduncle, trichome branching pattern on leaf margins, trichome branching pattern on tepals and 'spiky' fibrous sheath hairs presence), with 16 quantitative and 8 qualitative traits remaining (Table 3). All trichome traits removed had eigenvalues that were near 0 (i.e. 0.0000002) and were causing morphologically different individual to group together

incorrectly. Therefore, these traits were removed. A Principal Coordinates Analysis (PCoA) and Non-Metric Multidimensional Scaling analysis (NMDS) were conducted in R v3.4.3 (RStudio Team, 2015) using the package 'vegan' (Oksanen et al., 2022). The Gower general similarity coefficient was used for the PCoA as it is best suited for examining similarities and differences between individuals/varieties for morphometric data input (Gower, 1971). PCoA was used rather than a Principal Component Analysis (PCA) as the data set contains both qualitative and quantitative characters and a PCoA can be used with limited missing data (Borcard *et al.*, 2018). In addition, a PCA was conducted on the remaining quantitative data to generate eigenvalues and to assess the relative roles of quantitative vs. qualitative characters. Important distinguishing morphological features that had a high eigenvalue (shown via a PCA) were selected for direct comparison using box and whisker box plots to compare means ± standard errors (SE). An additional 35 individuals were collected from seven populations (Table 2) in South Africa and near the Lesotho border and were housed in the greenhouse at Wits University. Measurements of these individuals were compared separately to the herbarium specimens using box and whisker plots. The greenhouse and herbarium specimens were analysed separately as the morphology of the collected specimens changed slightly in greenhouse conditions. For example, many of the plants in the greenhouse had longer peduncles and leaves compared to the natural populations (and herbarium specimens), likely due to the lack of wind in the greenhouse. High eigenvalues indicate features that had a considerable influence in the directionality and positioning of the individuals on the principal axes. An ANOVA was conducted in R v3.4.3 (RStudio Team, 2015) to evaluate whether any significant differences exist in selected morphological features among, and between, the *Rhodohypoxis baurii* varieties. Two one-way ANOVAs were run; the first with variety as the fixed effect (testing if trends in morphological trait are due to variety) and the second with ploidy as the fixed variable (testing if trends in morphological traits are due to variety). Additionally a two-way multifactorial ANOVA was run with both ploidy and variety entered as explanatory variables. The results did not differ between the two types of ANOVA's. Tukey post-hoc analyses were performed on both ANOVA types to test for paired differences post ANOVA at a 95% confidence interval ($a = 0.05$).

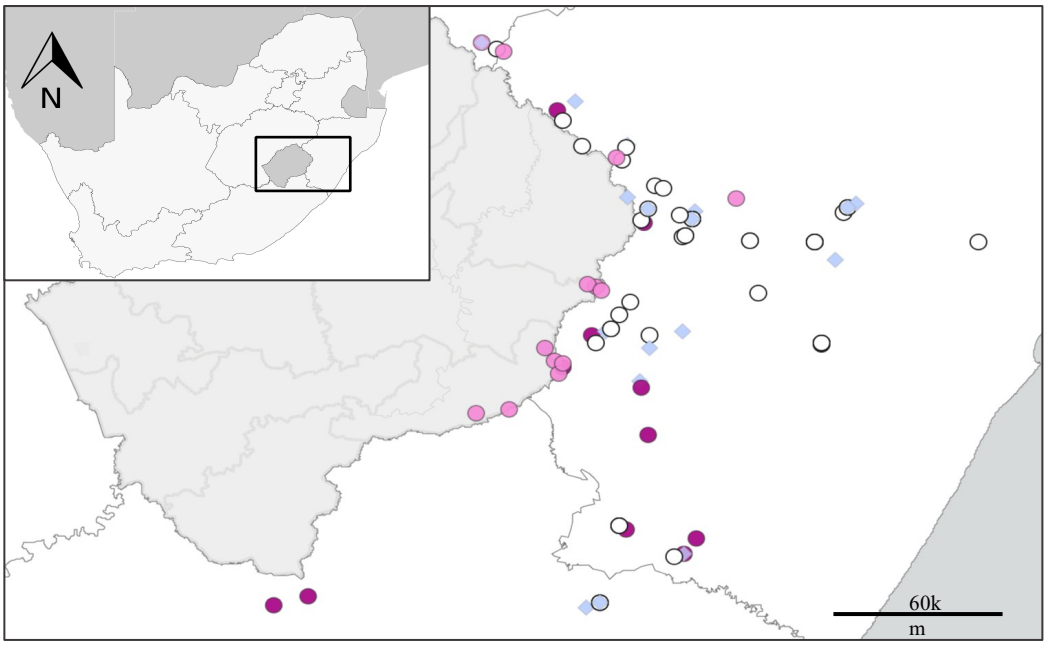

Figure 6. Geographic distribution of *Rhodohypoxis baurii* herbarium specimens used for morphometric analyses in this study. Dark pink: *Rhodohypoxis baurii* var. *baurii,* light pink: *Rhodohypoxis baurii* var. *confecta,* white: *Rhodohypoxis baurii* var. *platypetala* and pale blue: mixed individuals.

Flow cytometry was used to estimate the ploidy levels of 86 *R. baurii* var. *baurii* individuals, 30 *R. baurii* var. *confecta* individuals and 31 *R. baurii* var. *platypetala* individuals that were housed in the greenhouse. All individuals were housed in the same greenhouse under the same growth conditions. Greenhouse conditions attempted to mimic natural environmental conditions with the average summer temperatures ranging from 15°C to 30°C.. Flow cytometry provides an estimate of DNA content relative to a set standard from which ploidy level can be inferred. Flow cytometry was performed following Doležel et al., (2007) at the H.G.W.J. Schweickerdt Herbarium (PRU), University of Pretoria. *Oxalis articulata* Savigny genome size is known (pg = 1.96) and was used as the standard for flow cytometry on *Rhodohypoxis* individuals. Approximately 1 cm^2 of *O. articulata* and 1 cm^2 of *R. baurii* leaf tissue were finely chopped together using razor blades in 500 µl of Otto I lysis buffer. The solution was filtered through a 30 µm mesh filter to and incubated for 15 minutes at room temperature. One milliliter of Otto II buffer containing the fluorescent dye DAPI (4', 6-diamidino-2-phenylindole) was added to the solution to stain the cells and left to incubate at room temperature for a further 15 – 40 min. The samples were analysed using a CyFlowR Space flow cytometer. FloMax® software (Partec GmbH, 2001) was used to calculate the mean size of each peak (sample and

standard) and calculate the coefficient of variation. Relative DNA content was calculated. Ploidy levels were inferred by correlating the estimated DNA content to the base number of somatic chromosomes presented by Saito (1975).

Microsatellites Data Collection

Leaf material was collected from 237 individuals of *Rhodohypoxis baurii* (*R. baurii* var. *confecta* n = 88, *R. baurii* var. *baurii* n = *87*, *R. baurii* var. *platypetala* n = 62) across 11 populations (Figure 7; Table 2).

Table 2. Sampled localities, GPS co-ordinates and ploidy-levels present of 11 populations of *Rhodohypoxis baurii* varieties used in molecular microsatellite analyses and eight populations used in morphometric analyses (bolded/highlighted). No fresh leaf tissue was available for Sehlabethebe and Sani Chalet populations for flow cytometry, therefore ploidy levels were estimated based on microsatellite data (inferred from dosage). It is important to note that pure *R. baurii* var. *confecta* individuals are diploids only, but hybridization of this variety with other *Rhodohypoxis* species may lead to polyploid hybrids as observed in the Platberg population of *R. baurii* var. *confecta.*

Variety	Locality	Altitude (m a.s.l.)	GPS co-ordinates	Ploidy levels present
R. baurii* var. *baurii	**Naude's Nek**	**2400**	**-30.731515, 28.135147**	**Tetraploid (4×)**
R. baurii* var. *baurii	**Vrederus**	**1950**	**-30.790036, 28.262599**	**Tetraploid (4×)**
R. baurii var. *baurii*	Tenahead lodge	2500	-30.709617, 28.136370	Tetraploid (4×)
***R. baurii* var. confecta**	**Sentinel**	**2600**	**-28.751953, 28.890260**	**Diploid 2×**
***R. baurii* var. confecta (Hybrid population)**	**Platberg**	**2300**	**-28.245808, 29.161346**	**Diploid, triploid and tetraploid**
***R. baurii* var. platypetala**	**Golden Gate**	**2059**	**-28.517355, 28.616570**	**Diploid 2×**
R. baurii* var. platypetala & *R. baurii* var. *baurii	**Hebron Farm**	**1680**	**-33.441026, 150.868042**	**Diploid, Triploid and tetraploid**
***R. baurii* var. platypetala**	**Karkloof**	**1770**	**-29.298840, 30.269599**	**Diploid and tetraploid**
***R. baurii* var. platypetala**	**Sunset Farm**	**1934**	**-29.742937, 30.550833**	**Diploid, triploid and tetraploid**
R. baurii var. *baurii*	Sehlabethebe	2800	-29.824725, 29.200379	Diploids and polyploids
R. baurii var. confecta	Sani Chalet	2900	-29.585149, 29.288145	Diploids and polyploids

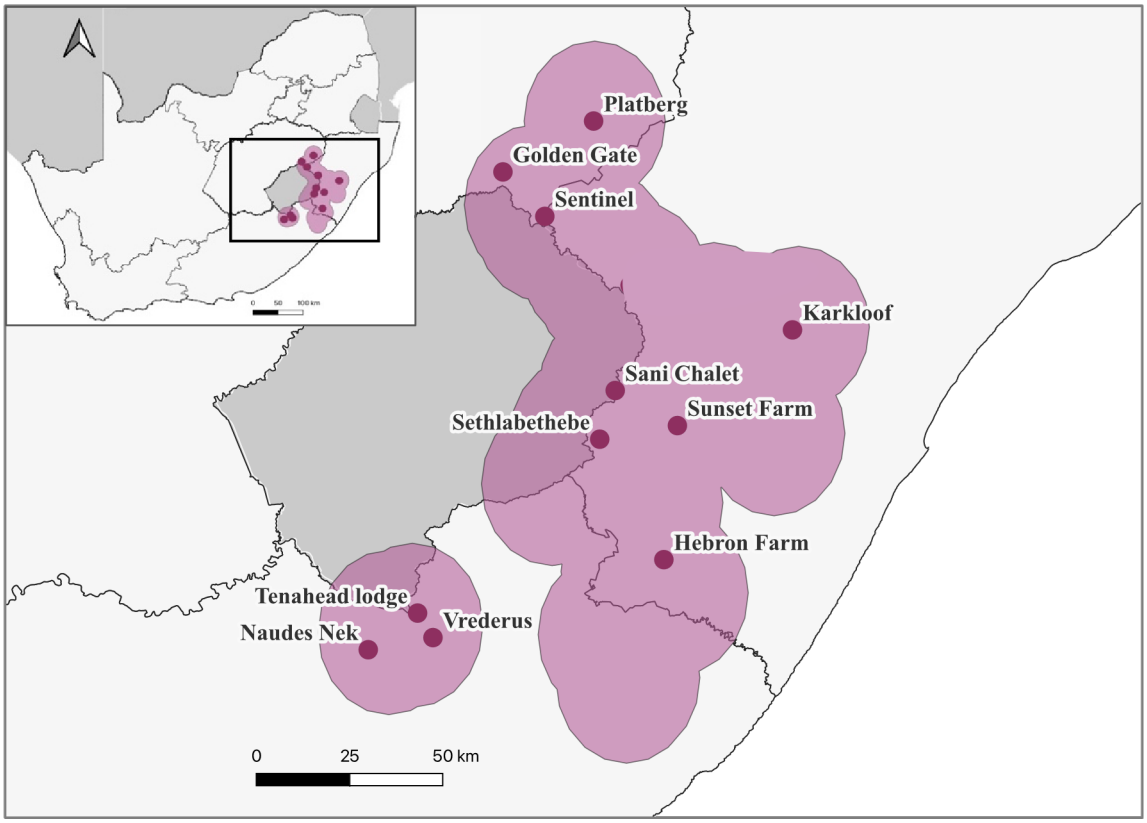

Figure 7. Distribution map of *Rhodohypoxis baurii* varieties with pink areas showing the known range of the varieties, and maroon points showing the 11 sampled populations.

The collected leaf material was dried in silica gel immediately to preserve the DNA for extraction. DNA was extracted from the sampled leaf material using a modified CTAB method following Doyle & Doyle, (1987) and Cullings, (1992). The CTAB extraction method was optimized for *Rhodohypoxis* with incubation times specified as follows. After addition of the CTAB buffer, the samples were incubated at 55°C for **90 minutes** and after the addition of isopropanol the samples were incubated at −80°C for **5 minutes** and then moved to −4 °C for an additional 15 minutes**.** These incubations times were optimal for large amounts of high-quality DNA with minimal contaminants that could inhibit PCR amplification. In the case of a very thin aqueous phase being present after the initial chloroform:isoamyl alcohol step, the chloroform:isoamyl alcohol step was repeated and samples re-centrifuged before pipetting off the aqueous phase. Ammonium acetate was excluded from this modified protocol. All DNA samples were quantified using a microvolume UV-Vis spectrophotometer (Nanodrop, Thermo Fisher Scientific) and good quality genomic DNA (A260/280 1.6 > 2.2) was diluted to a final concentration of 25–50 ng/µL before

amplification. Samples with low quality genomic DNA were re-extracted permitting enough leaf tissue remaining.

Sixty microsatellite markers were developed for *Rhodohypoxis* by AllGenetics (AllGenetics & Biology SL, A Coruña, Spain). The 60 primers developed were trialled and 28 amplified successfully; the 12 most variable markers were selected, optimized, and used in this study (Appendix, Table 2). The oligonucleotide tails used were the universal sequences CAG (CAG TCG GGC GTC ATC), and T3 (AAT TAA CCC TCA CTA AAG GG). The two oligonucleotides were labelled with the FAM dye, and the NED dye, respectively. Amplifications were performed using a single-plex fluorescent microsatellite PCR reaction. Amplifications were carried out using a BioRad T100™ thermal cycler using the following cycling profile: initial denaturation at 95 °C for 1 minute, followed by 33-35 cycles of 95 ºC for 20 s, 57 ºC for 60 s, 68°C for 45 s; and a final extension step at 68 ºC for 5 min. Success of the amplification was assessed via gel electrophoresis by mixing 3 µl 6x loading gel to 5 µl amplification reaction and loading samples onto a 2% agarose gel stained with SYBRSafe dye. The successfully amplified products were sent for fragment analysis at the Central Analytical Facility (CAF), Stellenbosch. The resulting chromatographs were scored manually. Peaks with a fluorescent intensity greater than 100 were scored and peaks with a fluorescent intensity less than 100 were removed. Distance between scored peaks was 4 base units or greater in order to avoid double scoring peaks that had stutters due to replication slippage.

A matrix was generated based on the scored peaks using the microsatellite plug in for Geneious Prime Version 11.0.6+10 (Biomatters Development Team, 2005). As the relative fluorescence of the peaks varied based on the success of the PCR reaction, dosage could not be inferred from the height (fluorescent intensity) of the peaks. Therefore, in all subsequent analyses using Genodive version 3.05 (Meirmans, 2020) the option to "correct for unknown dosage of alleles" was chosen. The method implemented by Genodive version 3.05 (Meirmans, 2020) to correct for unknown dosage assumes random mating within populations. The dosage correction uses a maximum likelihood statistical method based on random mating within populations. For every incomplete marker phenotype (e.g. AB) it is possible to calculate the likelihood of observing that phenotype given some set of allele frequencies, by combining the likelihoods of the underlying genotypes (AAAB, AABB, or ABBB)

(Meirmans, 2020). The algorithm starts by calculating the likelihood of all observed phenotypes for a population, given the uncorrected (biased) allele frequencies (Meirmans, 2020). The allele frequencies are then slightly changed and the likelihood is recalculated. If the fit improves, the changed allele frequencies are accepted and the old ones discarded (Meirmans, 2020). This process is continued until convergence is reached.

Microsatellites Data Analyses

Microsatellite data analyses were conducted to evaluate the extent of genetic differences and diversity between and within varieties, ploidy levels, and geographic populations. To compare genetic diversity both expected heterozygosity (H_E) and observed heterozygosity (Ho) were calculated using Genodive version 3.05 (Meirmans, 2020). Genetic distances, percentage polymorphic loci, Nei's gene distance index and inbreeding coefficient (F), were calculated and evaluated using Genodive (Meirmans, 2020). Any departures from HWE were assessed for each locus to ensure that the locus is not under selection and is suitable for further analyses. Nei's genetic distance was used to evaluate how genetically distinct the groups are and to what extent the taxa or geographic populations share alleles. The dataset was also evaluated for the presence of clones (see objective 3: Reproductive strategies) and clonal individuals were removed before performing population structure analyses.

The dataset contains mixed ploidy levels (diploids and polyploids). Therefore, the data were divided/ transformed to create five different data sets before performing population structure analyses. Firstly, the data set was divided into subsets based on ploidy levels, with diploids and tetraploids analysed separately, as well as together in a combined data set. The combined ploidy data set was further transformed with all diploids transformed into auto-polyploids by replicating the diploids alleles to create a polyploidized data set based on the maximum likelihood of different genotypes given the population allele frequencies (with the assumption of random mating within a population) (i.e. 170, 182, 0, 0 diploid genotypes were changed to 170, 182, 170, 182). Lastly, all polyploid individuals in the combined data set were made into diploids by removing alleles based on the most likely allele combinations using maximum

likelihood of different genotypes given the population allele frequencies with the assumption of random mating within a population. Removed alleles were made into a subsequent second individual, resulting in a diploidized data set. All data transformations were done in Genodive (Meirmans, 2020). Auto-polyploidizing the data set reinforces trends that already exist, but may reinforce trends that are not necessarily represented in the initial dataset. Therefore, it is important to perform analyses on the original data set as well as the transformed data sets to ensure trends stay the same and to ensure congruency and validity of the results.

Admixture (gene flow) was estimated and population structure was assessed using STRUCTURE 2.3.1 (Falush et al., 2007). STRUCTURE 2.3.1 uses a clustering method to assign individuals to clusters where Hardy-Weinberg Equilibriums (HWE) is achieved. To estimate K clusters, an admixture model with correlated allele frequencies was used, with an initial burn-in of 25 000, followed by 250 000 MCMC iterations. The K (possible number of genetic clusters) was set from one to 13 and with eight replicates per K value. K was set from one to 13 to accommodate the possible outcome of one genetic cluster (as they all belong to a single species) and the possible outcome of up to 13 genetic clusters due to the 11 populations present. The best fit value of K for the data was identified using STRUCTURE HARVESTOR (Earl & VonHoldt, 2011, https://taylor0.biology.ucla.edu/structureHarvester/) in order to identify the number of genetic clusters present and and whether these genetic cluster coincide with the recognised varieties, geographic populations, or ploidy. The proportion and pattern of shared alleles did not differ between the diploidized and polyploidized data sets, therefore the polyploidized data set was used for ordination and phylogenetic network analyses.

In the initial round of ordination analyses, two non-parametric methods were used to analyse the microsatellite data: Principal Component Analysis (PCA) and Discriminant Analysis of Principal Components (DAPC). While both PCAs and DAPCs provide a multivariate summary of genetic data, a DAPC also assesses the fit of data to a varying number of population clusters (Everson et al., 2021). Therefore, a DAPC yielded more accurate results for our data over a PCA. A Bruvo genetic distance matrix was generated using Genodive (Meirmans, 2020) and used to conduct a DAPC in R v3.4.3 (RStudio Team, 2015) using the package ‘adegenet’(Jombart, 2008). Lastly, the Bruvo genetic distance matrix was imported

into SplitsTree (Huson & Bryant, 2021) to generate a NeighborNet phylogenetic network and Neighbour Joining consensus tree to understand the relationships and reticulation events between and within the three varieties.

Reproductive strategies

To identify the breeding systems of *Rhodohypoxis baurii*, and to evaluate viability of crosses between varieties, greenhouse plants were divided into five experimental groups to test for the presence of the following: clonality, autonomous self-pollination, hand-manipulated self-pollination, cross-pollination by hand and agamospermy/apomixis.

Clonal reproduction

To check for and infer clonal reproduction, root connections were examined by uprooting plants that were originally planted as single individuals, but where the pots subsequently contained three or more individual stems within the same pot. Ten pots per variety (*R. baurii* var. *baurii, R. baurii* var. *confecta* and *R. baurii* var. *platypetala)* containing multiple plants were uprooted and the number of root connections recorded. From each of these pots, leaf tissue for three individuals per pot was collected for a total of 30 tissue samples per variety (90 tissue samples in total). DNA extractions and microsatellite amplification were performed (see 2.2 *Microsatellites Data Collection)* to confirm genetic similarity of these individuals. Genetic similarities and extent of clonality were identified using Genodive (Meirmans, 2020). If genetically identical, these individuals represent clonal reproduction. Uprooted plants were repotted with a single plant/bulb per pot. The pots were observed after one year and any new sprouts or root connections that formed were recorded.

Autonomous-self-pollination and Hand-self pollination

Five to fifteen greenhouse plants per variety were selected and bagged to test for autonomous self-pollination. All already open flowers were removed, and flower buds were enclosed in mesh organza bags. Hand self-pollinations were performed by removing all dehiscent anthers of a single flower and the pollen was applied to the stigma on the same flower by picking a dehiscent anther with forceps and gently dabbing the pollen onto the stigma, with care taken not to damage the stigma or tepals.

All flowers were then bagged using mesh organza bags and left to fruit. Seed set was recorded, and percentage of the seeds germinated was used to assess seed viability. Seed germination trials were carried out in the greenhouse. Seeds were placed in between pre-packaged sterile cotton wool that was dampened with autoclaved water, then placed in sealed plastic pre-packaged sterile containers and left in the greenhouse with average daytime temperatures between 25°C and 29°C under ambient light conditions. Seeds were checked weekly and all germinated seeds recorded. The germination trails were conducted for three consecutive months. Seeds that had not germinated after three months were marked as inviable. No tetrazolium salt staining was conducted on the seeds as the seeds were too small to yield accurate viability results from such staining techniques.

Cross-pollination viability

Ten to twenty plants of each variety were selected for cross-pollination by hand. Crosspollinated flowers were emasculated prior to the anthers maturing, with care taken not to transfer any pollen onto the stigma of the same plant. Pollen from a different variety was transferred onto the emasculated maternal plant's stigma for inter-varietal crosses, and pollen from the same variety was transferred onto the emasculated maternal plant's stigma for intravarietal crosses by picking a dehiscent anther with forceps and gently dabbing the open anther onto the stigma to transfer pollen, taking care not to damage the stigma or tepals. Intervarietal and intra-varietal crosses were performed between all three varieties, with at least 10 crosses representing each cross type; (cross types = b x c, b x p, c x p, c x c, b x b, p x p; Fig. 8). All flowers were bagged and left to set seed. Seed set, seed number, and seed viability (via germination) were recorded for each cross.

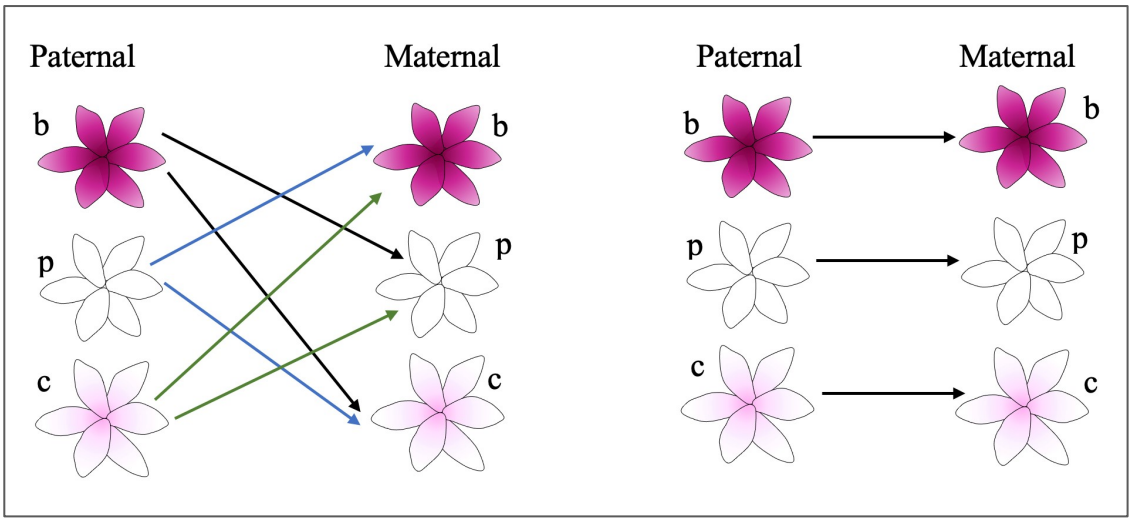

Figure 8. Both inter-varietal and intra-varietal crosses were conducted between the three *Rhodohypoxis baurii* varieties (b = *R. baurii* var. *baurii,* c = *R. baurii* var. *confecta,* p = *R. baurii* var. *platypetala*) with all varieties acting as both pollen donors (paternal) or pollen receivers (maternal). Arrows indicate direction of pollen transfer.

Agamospermy/apomixis

Ten to fifteen flowers of each variety were emasculated to test for agamospermy. After emasculation, all flowers were bagged using mesh organza bags and left to set seed. For all the above treatments, the number of seeds produced (if any) per plant/treatment were recorded and all seeds produced were germinated to test for viability.

Pollinator identification

In the field, pollinator observations were conducted at seven sites (Golden Gate National Park, Sentinel, Langalibalele Pass, Karkloof Nature Reserve, Minerva Reserve, Highmoor Nature Reserve and Vrederus Trout Farm). Pollinator observations were conducted between November and January 2021/2022 at the peak of the *Rhodohypoxis baurii* flowering season for one to two days per site. Observations were conducted for two continuous hours every six hours within a 24-hour period (i.e., for a total of eight hours within a 24-hour period), starting at approximately 6 am, 12 pm and 7 pm for the various observation sessions. Observations were conducted during both day and night hours. The frequency of insect visitation per plant and the type of visitation/ interaction was recorded and visiting insect(s) were caught when possible and placed in separate vials to avoid cross-contamination of pollen loads. The insects were identified to genus or species level using 'Field guide to insects of South Africa' by

Picker et al. (2004), and confirmed via consultation with entomologists. Pollen was washed off caught insects using ethanol and stained using Alexander's stain following Peterson et al.'s (2010) protocol. After staining, three to four drops of distilled water were used to rinse the slides and excess fluid absorbed with filter papers. Pollen slides were sealed and fixed using Hoyer's mounting medium (Anderson, 1954). Any pollen grains present were compared to *Rhodohypoxis baurii* pollen from greenhouse plants to ascertain if visiting insects were possibly acting as pollinators.

Pollen viability

In order to assess pollen viability, all anthers were removed from a single flower and were immediately placed into 1.5 ml microcentrifuge tubes containing Carnoy's Fixative to fix the pollen. The top three anthers (top whorl) and bottom three anthers (bottom whorl) were collected into separate microcentrifuge tubes as it was hypothesized that the whorls may mature at different times. The anthers and pollen were left in the Carnoy's Fixative for a minimum of 48 hours and a maximum of four months. Thereafter, both the top and bottom anthers were removed, and pollen was stained using Alexander's stain following Peterson et al.'s (2010) protocol. After staining, three to four drops of distilled water were used to rinse the slides and excess fluid absorbed with filter paper. Pollen slides were sealed and fixed using Hoyer's mounting medium. The numbers of viable (red) and inviable (green) pollen grains (Fig. 9) were counted per slide using a Zeiss compound light stereo-microscope at 100× magnification.

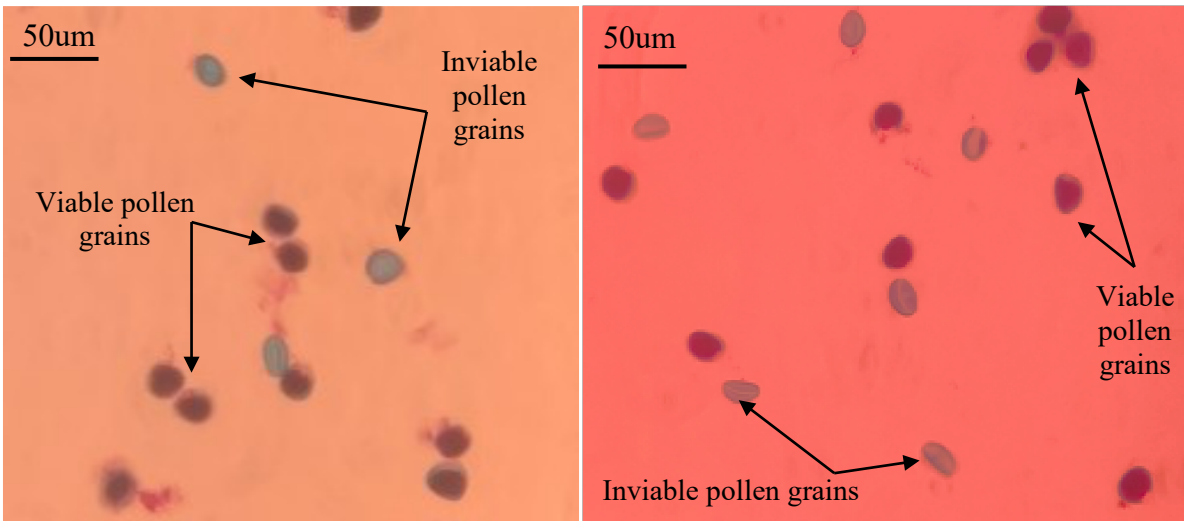

Figure 9. *Rhodohypoxis baurii* pollen grains stained with Alexander's stain showing viable pollen (red) and inviable pollen (green/blue).

CHAPTER 3: RESULTS

Morphometric analysis of R. baurii varieties and ploidy levels

Of the 126 herbarium specimens selected for morphometric analyses, 10 specimens were removed from the data set as they were identified as hybrid individuals (as they fell far outside the morphometric space of the other individuals/ known varieties) and were from localities where high levels of introgression and hybridization between *R. baurii* and other *Rhodohypoxis* or *Hypoxis* species were noted by Hilliard & Burtt (1978). A total of 8 traits were removed (tufted trichomes on tepal tips, peduncle trichome length, tepal trichome length, trichome density on peduncle, trichome branching pattern peduncle, trichome branching pattern on leaf margins, trichome branching pattern on tepals and 'spiky' fibrous sheath hairs presence), with 16 quantitative and 8 qualitative traits remaining (Table 3). Clear overlap of the specimens in the ordination analyses meant that little morphological distinction was found between the three varieties (Fig. 10), when qualitative data were excluded, no separation of the varieties was found with all three varieties overlapping completely. *Rhodohypoxis baurii* var. *platypetala* shows the largest morphological variation and overlaps mainly with *R. baurii* var. *confecta* to some extent, and *R. baurii* var. *baurii* and *R. baurii* var. *confecta* overlap considerably, indicating that they are less morphologically distinct from each another. Three outlier individuals from the Northern and Central Drakensberg were identified in the *R. baurii* var. *confecta* group

(Hc113, Hcpv122 and Hcm114; Fig. 10) that were collected from Cathkin Peak, Mont Aux Sources and Cathedral Peak, respectively. These three localities have multiple *Rhodohypoxis* and *Hypoxis* species present with introgression and hybridisation possibly occurring.

Table 3: List of contributions (loadings) of each morphological character measured for the first four Principal Component Axes, based on the PCA analysis.

	PC 1	PC 2	PC 3	PC 4
Peduncle Length (mm)	**0.934**	**-0.357**	-0.007	-0.002
Leaf length	**0.897**	**0.441**	-0.001	0.002
Leaf width	0.220	**-0.298**	0.262	**0.713**
Peduncle trichome length	0.390	0.002	0.214	0.417
Ovary trichome length	0.328	0.088	0.191	0.369
Leaf trichome length	0.331	-0.046	0.045	**0.520**
Tepal Trichome length	0.338	0.131	0.181	0.313
# Flowers/ peduncle	0.231	-0.081	-0.016	-0.127
Outer tepal length	0.569	-0.040	**0.787**	-0.146
Outer tepal width	0.358	-0.161	**0.763**	0.038
Outer tepal ratio	-0.103	0.232	-0.080	0.177
Trichome density peduncle	-0.155	0.132	0.148	0.188
Trichome density leaf	**-0.357**	-0.182	0.054	0.014
Trichome density ovary	0.251	-0.065	-0.015	0.281
Trichome density tepal	-0.0689	-0.155	0.145	0.157
Trichome branching pattern peduncle	**-0.202**	-0.065	0.113	-0.086
Trchome branching pattern leaf centre	0.013	0.167	**-0.135**	-0.683
Trichome branching pattern leaf margin and midrib	0.129	-0.013	-0.030	-0.011
Trichome branching pattern tepal	0.072	-0.136	0.005	**-0.163**
Flower colour (as noted by collector)	-0.127	**0.341**	**-0.316**	0.192
Spiky sheaths	-0.141	-0.099	0.051	-0.033
Tufted trichomes on tepal tips	0.025	-0.028	-0.024	0.153
Trichome density pattern on leaves	-0.175	-0.197	0.278	**-0.242**

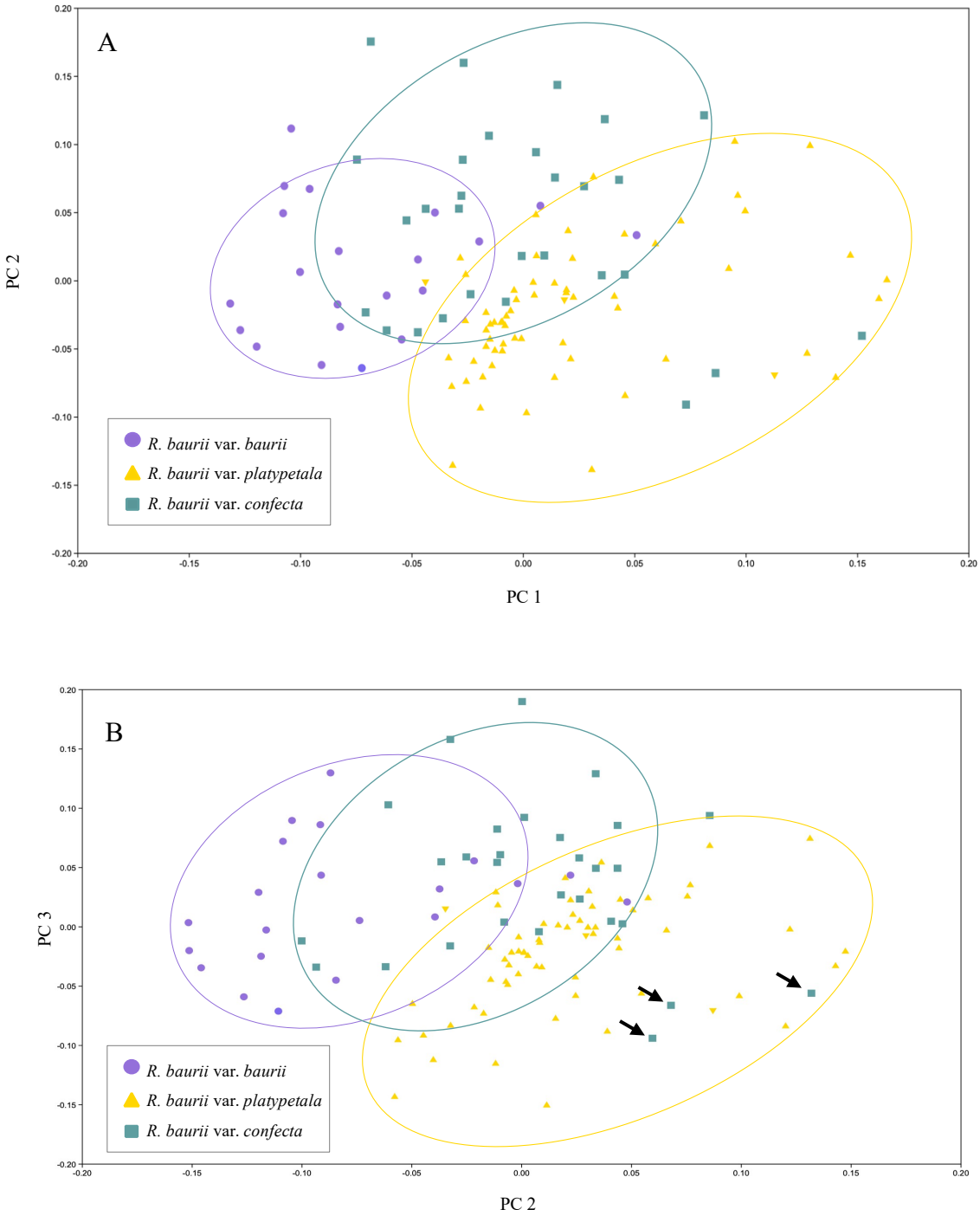

Figure 10. Non-metric multidimensional scaling (NMDS) analysis of 116 herbarium specimens of *R. baurii* varieties based on 23 morphological traits using the Gower similarity coefficient. A) PC axes 1 and 2 explain 65% of the variation, B) PC axes 2 and 3 explain 54% of the variation. Three outliers of *R. baurii* var. *confecta* (arrowed) showed traits commonly seen in *Hypoxis* species: Hcm104 from Cathedral Peak; Hc113 from Monks Cowl Reserve and Hcpv122 from Mont Aux Sources.

Distinguishing morphological features and those with high eigenvalues were selected for direct comparison using box and whisker box plots (Fig.11–15). Due to the polyploid nature of the sampled *R. baurii* individuals, it is important to note that population ploidy levels may have changed over time, and changes in ploidy may lead to morphological changes and shifts. Therefore, herbarium specimens and greenhouse individuals were analysed separately. Trichome traits such as the length of trichomes present on the peduncle of herbarium specimens did not differ significantly between varieties (df = 108, F = 1.59, P = 0.21*)* and leaf lamina length did not differ significantly between varieties (df = 108, F = 2.18 P = 0.12) (Fig. 11). Leaf widths of *R. baurii* var. *baurii* herbarium specimens was significantly smaller than those of *R. baurii* var. *confecta* (df = 105, F = 3.41 P = 0.032) and *R. baurii* var. *platypetala* (df = 105, F = 3.41, P = 0.012) (Fig. 11). Certain traits of *Rhodohypoxis baurii* var. *platypetala* specimens were significantly larger than in *R. baurii* var. *baurii* and *R. baurii* var. *confecta*, such as peduncle length (df = 108, F = 8.03, P = 0.00056), outer tepal width (df = 100, F = 8.37, P = 0.00043) and outer tepal length (df = 100, F = 10.48, $P < 0.001$) (Fig. 11).

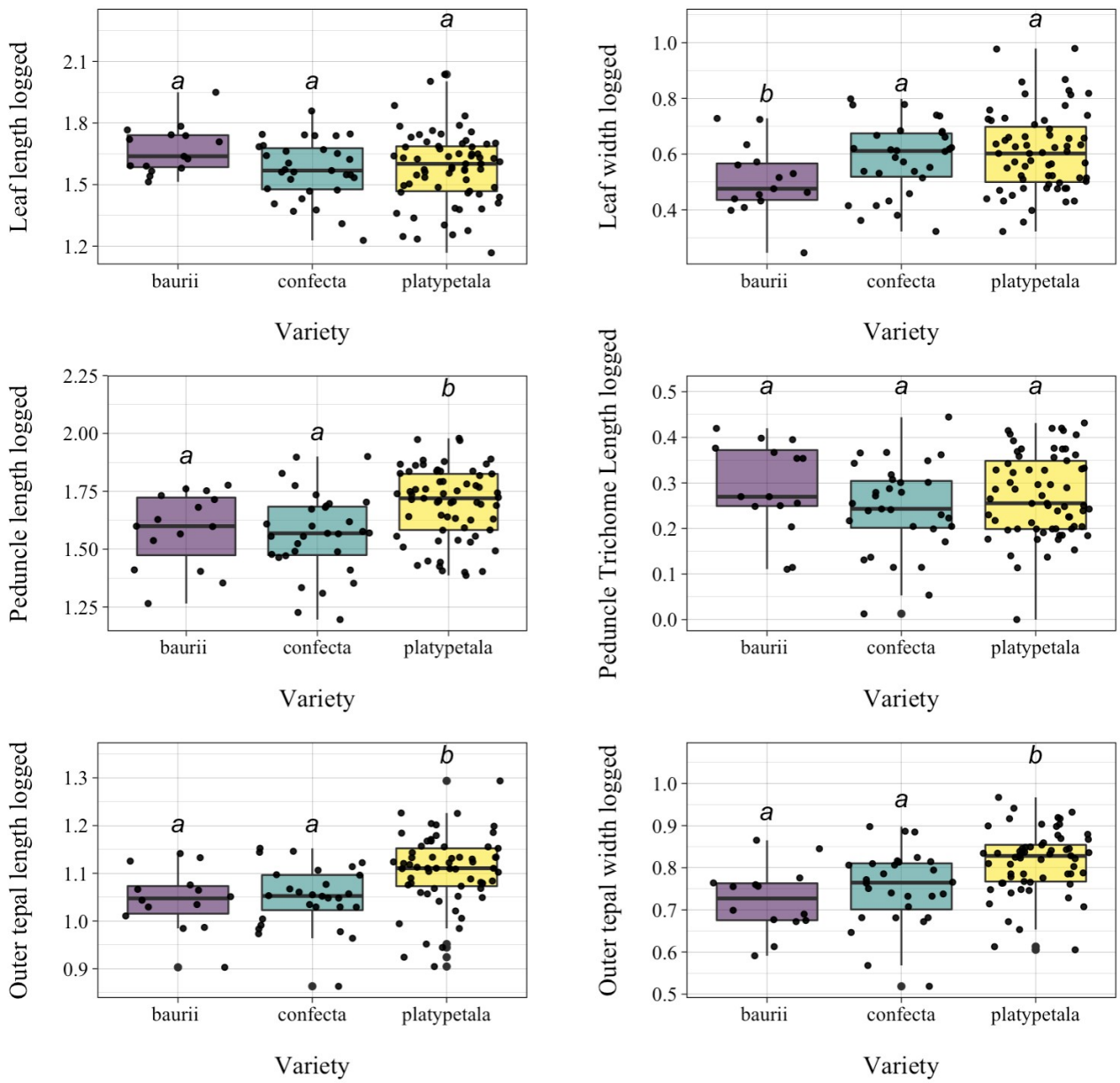

Figure 11. Comparison of vegetative, and floral morphological traits of 116 herbarium specimens represented by *Rhodohypoxis baurii* varieties: baurii = *Rhodohypoxis baurii* var. *baurii*, confecta = *Rhodohypoxis baurii* var. *confecta* and platypetala = *Rhodohypoxis baurii* var. *platypetala.* All measurements were log transformed and significant differences are indicated above each box to a 95% confidence interval (α = 0.05). The compact letter display is based on Tukey Posthoc tests derived from two-way ANOVAs.

Rhodohypoxis baurii individuals housed in the greenhouse were collected throughout the Drakensberg region between 2017 and 2020 and all measurements were conducted in 2021. A total of seven vegetative, peduncle and trichome traits were measured across 43 greenhouse individuals, and 12 floral traits measured across 31 greenhouse individuals. *Rhodohypoxis baurii* var. *platypetala* has significantly larger floral features than *R. baurii* var. *baurii* and *R.*

baurii var. *confecta*, such as outer tepal width (df = 26, $F = 27.69$, $P < 0.001$), inner tepal length (df = 26, $F = 8.09$, $P = 0.0019$) and inner tepal width (df = 26, $F = 6.39$, $P = 0.0055$), but has significantly shorter leaves than *R. baurii* var. *confecta* (df = 21, $F = 3.66$, $P = 0.043$) (Figs.12, 13). *Rhodohypoxis baurii* var. *platypetala* and *R. baurii* var. *baurii* have, on average, similar leaf widths (df = 21, $F = 3.66$, $P = 0.24$), whereas *R. baurii* var. *platypetala* has significantly wider leaves than *R. baurii* var. *confecta* (*df* = 21, $F = 3.96$, $P = 0.028$) (Fig. 13). *Rhodohypoxis baurii* var. *baurii* and *R. baurii* var. *confecta* do not differ in most floral size measurements, including outer tepal length (df = 26, $F = 3.81$, $P = 0.90$), inner tepal length (df = 26, $F = 8.09$, $P = 0.75$) and inner tepal width (*df* = 26, $F = 6.39$, $P = 0.50$) (Fig. 12). Female reproductive traits do not differ between the three varieties, such as pistil length (df = 26, $F = 6.39$, $P = 0.309$) and ovule width (df = 29, $F = 0.76$, $P = 0.48$) (Fig. 12). On the other hand, male reproductive traits namely anther length and anther width do differ, where *R. baurii* var. *platypetala* has significantly longer anthers than *R. baurii* var. *baurii* (df = 29, $F = 2.35$, $P = 0.096$) (Fig. 12; Fig. 14). Flower colour is one of the main distinguishing features used to identify *R. baurii* varieties, with *R. baurii* var. *baurii* exhibiting dark pink tepals, R. *baurii* var. *confecta* flowers showing various shades of white and pinks, and *R. baurii* var. *platypetala* showing exclusively white flowers with the occasional pink flushes on tepal tips (Fig. 14; Fig. 15).

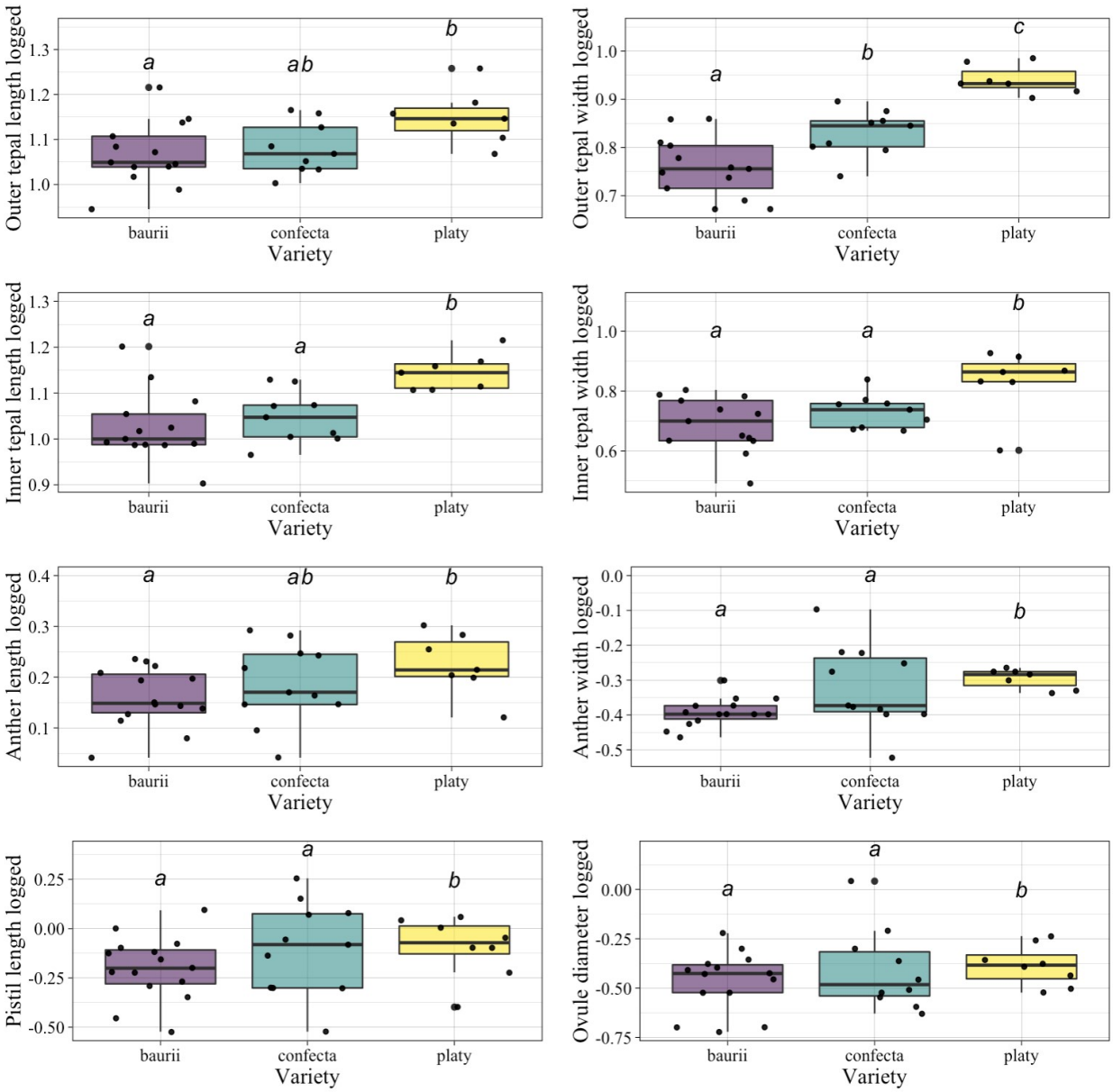

Figure 12. Comparison of floral morphological traits of 31 collected greenhouse specimens represented by *Rhodohypoxis baurii* varieties: baurii = *Rhodohypoxis baurii* var. *baurii*, confecta = *Rhodohypoxis baurii* var. *confecta* and platy = *Rhodohypoxis baurii* var. *platypetala.* All measurements are log transformed and significance levels are indicated above each box to a 95% confidence interval (α = 0.05). The compact letter display is based on Tukey Posthoc tests derived from two-way ANOVAs.

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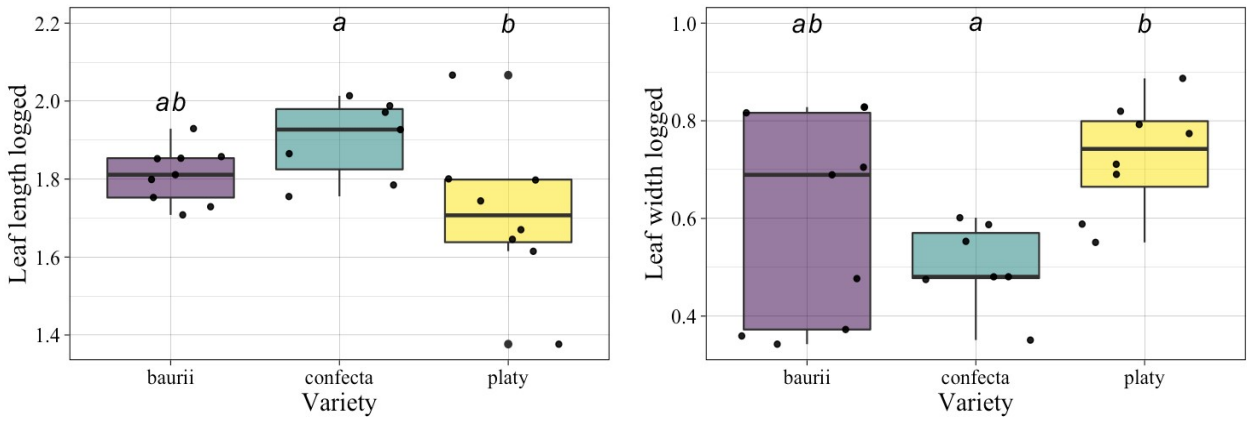

Figure 13. Comparison of vegetative morphological traits of 43 herbarium specimens represented by *Rhodohypoxis baurii* varieties with baurii = *Rhodohypoxis baurii* var. *baurii*, confecta = *Rhodohypoxis baurii* var. *confecta* and platy = *Rhodohypoxis baurii* var. *platypetala.* All measurements were log transformed and significance levels are indicated above each box to a 95% confidence interval (α = 0.05). The compact letter display is based on Tukey Posthoc tests derived from two-way ANOVAs.

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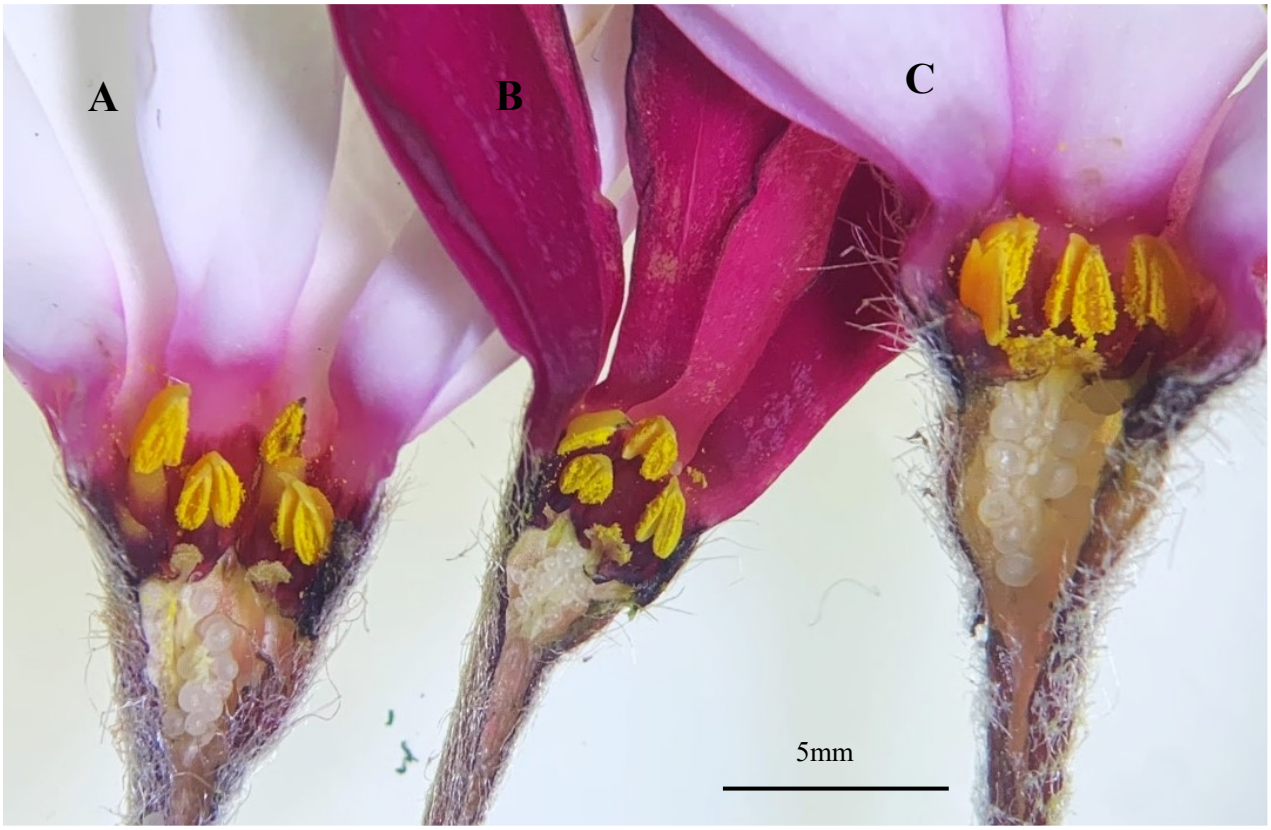

Figure 14. Image showing the size difference of reproductive organs such as anthers and ovules between (A) *Rhodohypoxis baurii* var. *platypetala,* (B) *Rhodohypoxis baurii* var. *baurii,* (C) *Rhodohypoxis baurii* var. *confecta.*

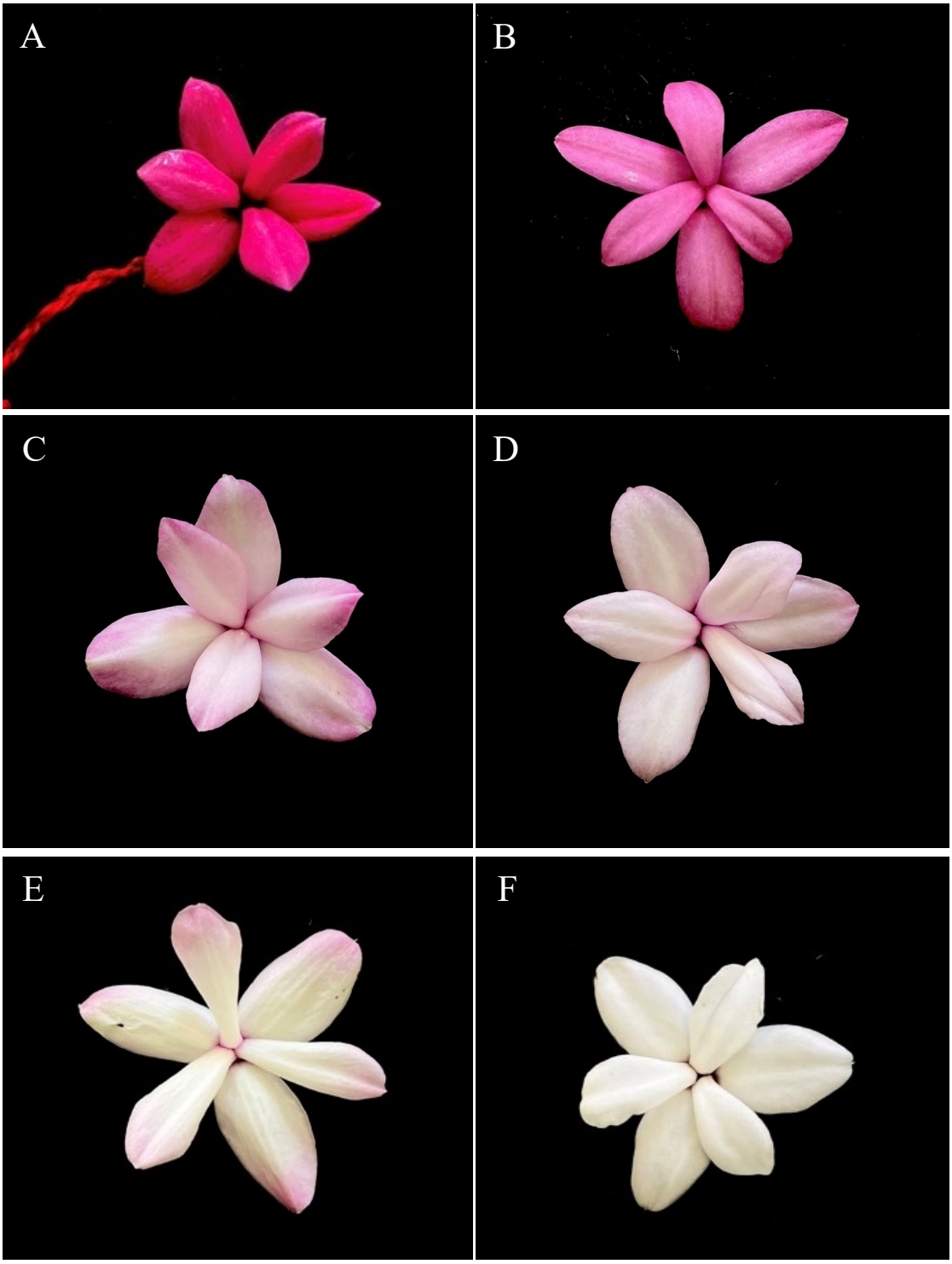

Figure 15. Variation in flower colour of *R. baurii* individuals housed in the greenhouse: A) *R. baurii* var. *baurii* from the Vrederus population, Eastern Cape Drakensberg; B) *R. baurii* var. *baurii* from the Hebron Farm population, Southern Drakensberg, exhibiting dark pink tepals; C and D) R. *baurii* var. *confecta* flowers from the Sentinel population, Northern Drakensberg, showing various shades of white and pinks; E) *R. baurii* var. *platypetala* from Sunset Farm population (away from the main Drakensberg) showing white flowers with the occasional pink flushes on tepal tips; F) exclusively white flowered *R. baurii* var. *platypetala* from Karkloof, which region (away from the main Drakensberg). Images taken in 2021.

Rhodohypoxis baurii varieties exhibit multiple ploidy levels (Table 2; Appendix, Table 4), and changes in ploidy may lead to morphological shifts, therefore, morphological traits were grouped by ploidy level to test if morphological variation or differentiation is due to ploidy level. Only three triploids were present in the dataset and were excluded due to the low sample size. Leaf length did not differ between diploids and tetraploids (df = 22, $F = 0.72$, $P = 0.41$) nor did leaf width (df = 22, $F = 0.82$, $P = 0.38$) (Fig.17). However, there are two distinct groups present in the tetraploid leaf width measurements. The first group comprises *R. baurii* var. *baurii* individuals from the Eastern Cape Drakensberg, noted by Hilliard & Burtt (1978, p.57) as the "on the face of the main Berg at heights". In this dataset, these individuals consisted exclusively of tetraploids (Table 2; Appendix, Table 4). The second group consists of *R. baurii* var. *baurii "*away from the main Drakensberg" individuals and *R. baurii* var. *platypetala* polyploid individuals. The "on the face of the main Berg" *R. baurii* var. *baurii* tetraploid individuals' leaves are 57.95% narrower (2.52 mm ± 0.24) than *"*away from the main Drakensberg" *R. baurii* var. *baurii* diploid individuals (5.99 mm ± 0.416). There is no significant difference in leaf widths between diploids and tetraploids (df = 22, $F = 0.82$, $P = 0.38$), but tetraploid individuals exhibit greater variation than diploids (Fig. 17). Diploids and tetraploids also do not differ in inner tepal width (df = 27, $F = 0.20$, $P = 0.66$), outer tepal widths (df = 27, $F = 0.42$ $P = 0.52$, Fig. 16), pistil length (df = 27, $F = 1.93$, $P = 0.33$) and ovule size (df = 29, $F = 0.78$, $P = 0.48$) (Fig. 16). However, anther length (df = 30, $F = 7.04$, $P = 0.012$). anther width (df = 30, $F = 4.65$, $P = 0.039$), outer tepal length (df = 27, $F = 0.43$, $P = 0.032$) and inner tepal length (df = 27, $F = 0.52$, $P = 0.024$) are significantly larger in tetraploid individuals than diploids (Fig. 16).

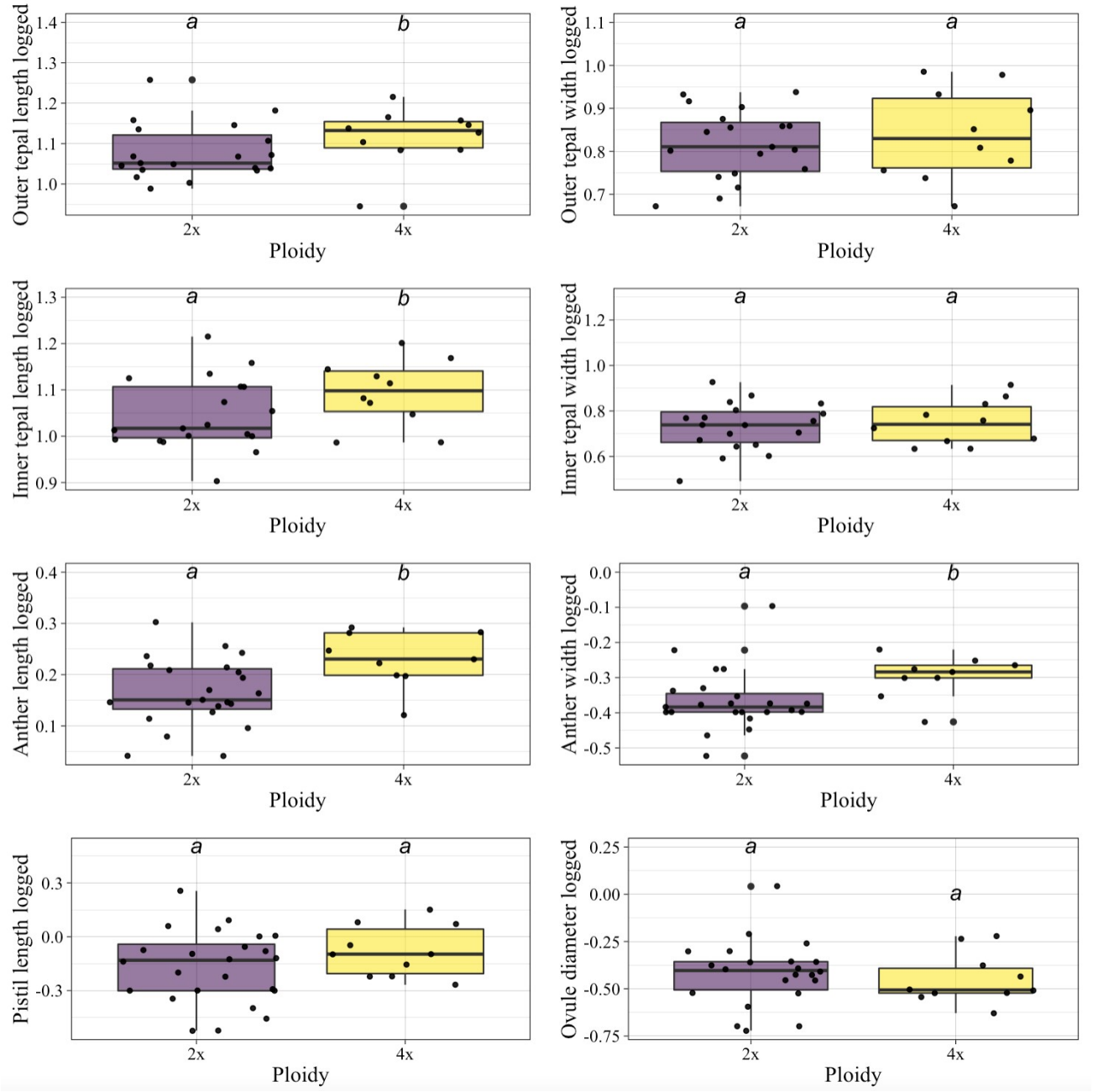

Figure 16. Comparison of floral morphological traits across 31 collected greenhouse specimens of *Rhodohypoxis baurii* varieties grouped by ploidy level : 2× = diploids and 4× = tetraploids. All measurements were log transformed and significant differences are indicated above each box to a 95% confidence interval (α = 0.05). The compact letter display is based on Tukey Posthoc tests.

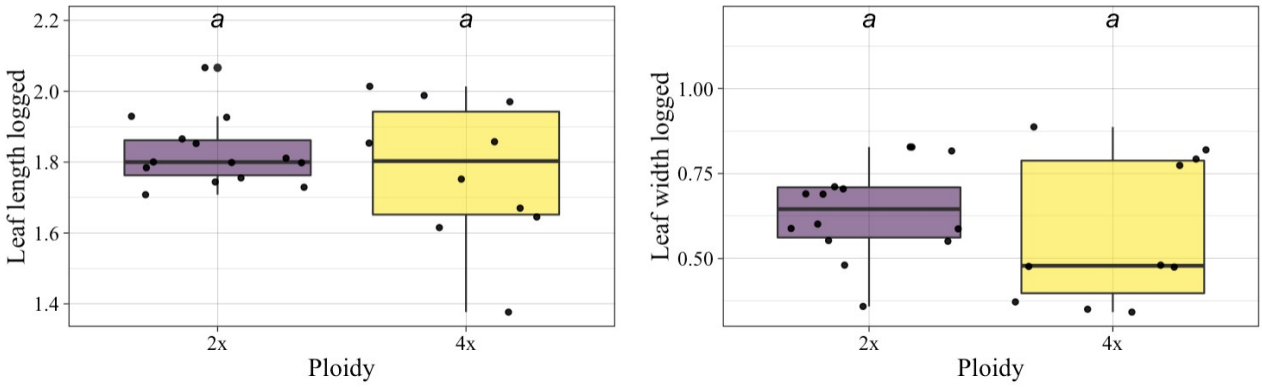

Figure 17. Comparison of vegetative morphological traits of 31 collected greenhouse specimens of *Rhodohypoxis baurii* varieties grouped by ploidy level : 2× = diploids and 4× = tetraploids. All measurements were log transformed and significant differences are indicated above each box to a 95% confidence interval (α = 0.05). The compact letter display is based on Tukey Posthoc tests.

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The *R. baurii* varieties occur at different altitudes: *R. baurii* var. *platypetala* occurs at lower altitudes (< 2200 m a.s.l) and *R. baurii* var. *confecta* occurs at higher altitudes (2200–3000 m a.s.l). Therefore, 10 quantitative morphological traits were correlated with altitude to test if morphological differences are a function of altitude and not due to taxonomy or ploidy level. There was no significant relationship between any quantitative morphological trait and altitude (Appendix, Figure 3).

Microsatellite analyses - Genetic differentiation and gene flow

Microsatellite analyses were conducted on 237 individuals using 12 microsatellite markers which yielded a total of 221 alleles. The total number of alleles detected per locus ranged from nine alleles in the loci AG_Rba_304 and AG_Rba489 to 36 alleles in the locus AG_Rba_307 (Appendix, Table 5). The error rate calculated from the replicates was less than 0.01%. All clones were identified, and 88 clonal individuals removed from the data set before genetic diversity and genetic structure were calculated. 149 of 237 individuals were evaluated for genetic diversity (Table 4). The observed number of alleles (Ao, the actual number of alleles found in our sampled population) was calculated as well as the effective number of alleles (Ae, the number of equally frequent alleles that it would take to achieve the same expected heterozygosity in the sampled population). The effective number of alleles for the entire data set was lower than the observed number of alleles (Ae = 3.161 < Ao =

18.417, Table 3.). Observed heterozygosity (Ho) was lower than the expected heterozygosity (Ht) (0.491 < 0.766) (Table 4). Genetic diversity did not vary greatly between populations, varieties and ploidy levels. Observed heterozygosity for populations ranged from 0.62 – 0.74 (Table 4). Naudes Nek and Platberg populations showed the highest heterozygosity (Hs = 0.74 and Hs = 0.71 respectively; Table 3). Sunset Farm, Sehlabethebe, Hebron Farm and Golden Gate showed the lowest heterozygosity (Hs = 0.62–0.65) (Table 4). Heterozygosity did not differ significantly between the ploidy levels with triploids showing the lowest heterozygosity (Hs = 0.71) and diploids with the highest heterozygosity (Hs = 0.79). Similarly, heterozygosity did not differ significantly between the varieties (Hs = 0.70 – 0.76; Table 4). All populations showed some inbreeding, with Golden Gate and Sentinel populations showing the highest inbreeding values (Gis = 0.46 and Gis = 0.38 respectively; Table 4). Tenahead Lodge, Platberg and Vrederus had the lowest inbreeding values (Gis = 0.086 – 0.18; Table 4), however, this value may not be accurate for Platberg due to the low sample size for the Platberg population (n = 3). Inbreeding did not differ greatly between ploidy levels (Table 4). Triploids had the least inbreeding (Gis = 0.24) and diploids had the most inbreeding (Gis = 0.38; Table 4). The varieties also did not show large differences in inbreeding across all populations (Gis = 0.27 – 0.39).

Genetic differentiation showed that geographically closer populations have lower Nei's Gst genetic differentiation irrespective of the variety (Appendix, Table 6). Sani Chalet, Sehlabethebe, Sunset Farm and Hebron Farm showed low genetic differentiation to one another (Nei Gst = 0.054 – 0.075; Appendix, Table 6) and are in close geographic proximity, have similar genetic/ allelic structures and are all populations comprising individuals of mixed ploidy levels (i.e., each population has more than one cytotype present) (Fig. 18). Yet, these four populations consist of different varieties. Naudes Nek, Vrederus and Tenahead Lodge populations were genetically similar to each other (Nei Gst = 0.06 – 0.104) (Appendix, Table 6) and are somewhat geographically isolated in the Eastern Cape Drakensberg. Naudes Nek, Vrederus and Tenahead Lodge populations are geographically close, and share alleles resulting in similar genetic profiles and consist entirely of tetraploid individuals and all consist of a single variety, *R. baurii* var. *baurii* (Fig. 18). The Northern Drakensberg populations, Golden Gate *R. baurii* var. *platypetala* and Platberg *R.*

baurii var. *confecta*, show the highest genetic differentiation between each other (Nei Gst = 0.21 respectively) (Appendix, Table 6).

Table 4. Indices of genetic diversity based on 12 microsatellite markers across 11 sampled populations, three varieties (*Rhodohypoxis baurii* var. *baurii*, *Rhodohypoxis baurii* var. *confecta* and *Rhodohypoxis baurii* var. *platypetala)* and three ploidy levels (2×, 3× and 4×) in *Rhodohypoxis baurii* varieties with clonal individuals (n = 88) removed. N_{ind} = number of individuals, Ao = the observed number of alleles, Ae = effective number of alleles, Ho = observed individual heterozygosity, Hs = heterozygosity in population, also known as gene diversity, G_{is} = inbreeding coefficient.

		N_{ind}	**Ao**	**Ae**	**Ho**	**Hs**	**G_{is}**
Populations	Golden Gate	7	4.25	3.07	0.35	0.65	0.46
	Platberg	3	4.25	3.43	0.59	0.71	0.17
	Sentinel	15	7.58	3.32	0.43	0.69	0.38
	Hebron Farm	14	8.08	3.23	0.44	0.65	0.33
	Sani Chalet	19	7.42	4.11	0.44	0.65	0.32
	Sehlabethebe	22	7.83	3.67	0.42	0.64	0.34
	Sunset Farm	4	4.08	2.67	0.43	0.62	0.31
	Tenahead Lodge	9	7.25	4.35	0.64	0.70	0.09
	Naudes Nek	19	9.50	4.69	0.59	0.74	0.21
	Vrederus	17	7.92	3.68	0.57	0.70	0.18
	Karkloof	20	6.25	3.11	0.50	0.62	0.2
	All populations	149	18.42	3.16	0.49	0.67	0.27
Ploidy levels	Diploid	26	13.33	5.48	0.49	0.79	0.38
	Triploid	35	10.25	4.12	0.54	0.71	0.24
	Tetraploid	88	17.50	5.3	0.56	0.76	0.26
Varieties	var. *baurii*	56	13.50	5.21	0.55	0.76	0.27
	var. *confecta*	59	13.08	4.73	0.44	0.72	0.39
	var. *platypetala*	34	9.08	3.93	0.47	0.70	0.33

The STRUCTURE analysis of the complete matrix comprising 149 individuals and 221 alleles from 12 microsatellite markers suggested that the data represent four clusters (Fig. 18A). The trends did not differ between the diploidized and

polyploidized data sets (Appendix, Figure 5), therefore the polyploidized data set results are presented. Four genetic clusters were identified as optimal; however, six genetic clusters and 11 genetic clusters were also likely (Appendix, Figure 5). The clustering corresponded with geographic localities and not taxonomic classification, except for Platberg and Karkloof localities which showed unique genetic profiles that did not follow geographic or taxonomic patterns (Fig. 18B). Individuals from geographically close populations share the same alleles and show similar trends in ploidy levels, with the Northern Drakensberg populations, Golden Gate and Sentinel (excluding Platberg) consisting entirely of diploid individuals, while the Eastern Cape Drakensberg populations (Tenahead Lodge, Vrederus and Naude's Nek) consist exclusively of tetraploid individuals (Fig. 18B). Populations in the Southern Drakensberg (Sani Chalet, Sunset Farm, Sethlabethebe, Hebron Farm) showed nearly identical genetic profiles, sharing the same alleles, and all consisted of mixed ploidy populations (i.e., diploids, triploids and/or tetraploids co-occurring in the same population; Fig. 18B). The NeighborNet phylogenetic network clustering was consistent with this genetic structure (Fig.18C). Furthermore, a latitudinal ploidy gradient is observed across the sampled populations. Diploids appear to occur primarily in the Northern Drakensberg at the more northerly latitudes (ca. 28 ° S) and polyploids occur primarily in the Eastern Cape Drakensberg at the more southerly latitudes (ca. 30° S), and there is a transitional area in between these two geographic cytotype zones (ca. 29 ° S) where mixed ploidy populations tend to occur (Fig. 18b). An AMOVA revealed that the differentiation between varieties explained only 5.4% of variance, while differentiation between populations explained 24.7% of variance and differentiation between ploidy levels explained 6.1%. Most variation is present within individuals (64.2%) and among individuals (26.3%).

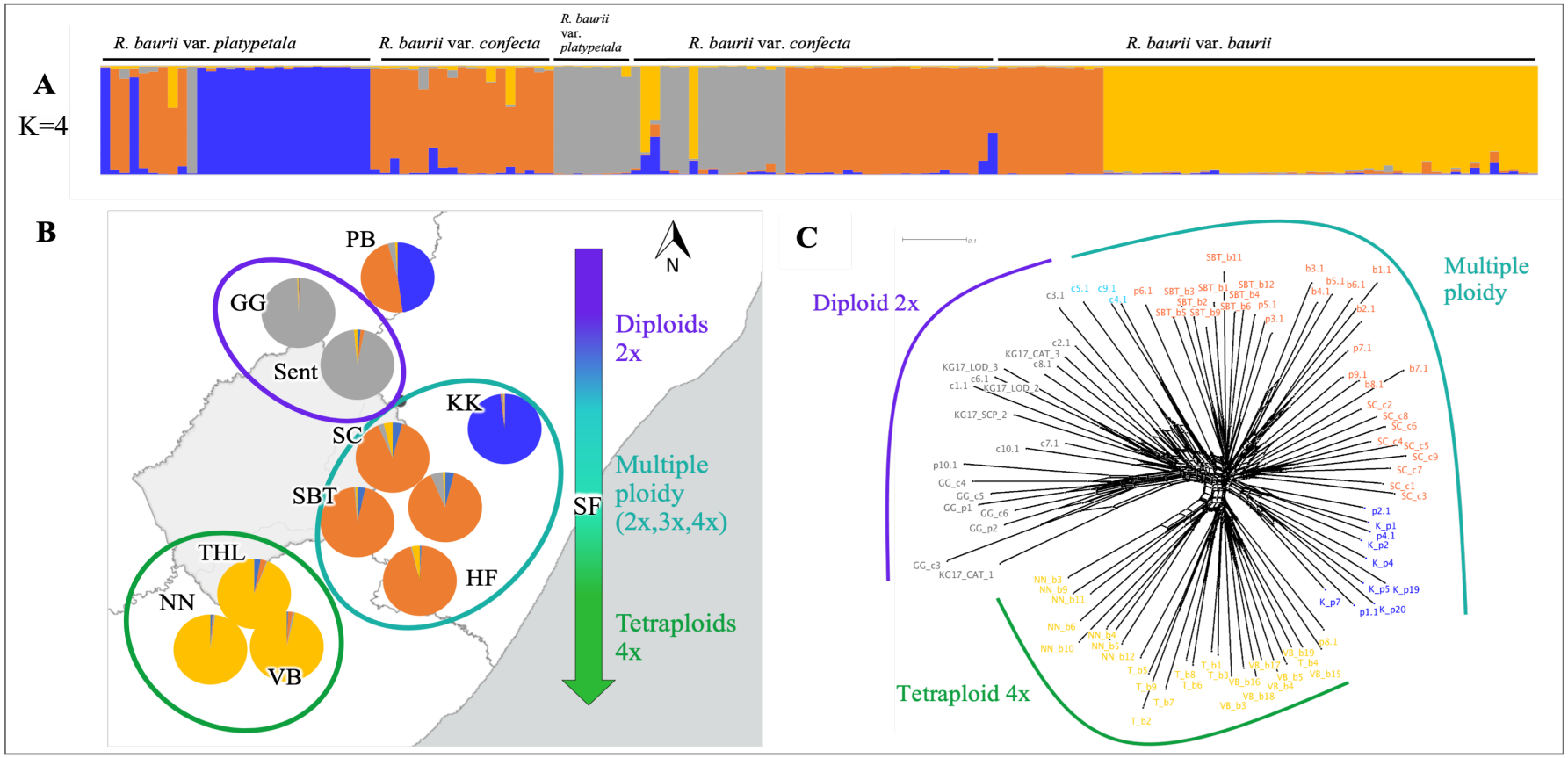

Figure 18. Population genetic structure, allelic diversity and ploidy of the *R. baurii* populations based on auto-polyploidized data from 12 microsatellite markers. **A)** Bayesian model-based clustering method implemented in STRUCTURE showed K = 4 best represented the data. The colouring of vertical bars indicates each individual's proportional cluster assignment. **B)** Distribution of the sample sites: PB = Platberg, GG = Golden Gate, Sent = Sentinel, KK= Karkloof, SC = Sani Chalet, SBT = Sethlabethebe, SF = Sunset Farm, HF = Hebron Farm, THL = Tenahead lodge, NN = Naudes Nek, VB = Vrederus. Different coloured pie charts show the population assignment to four genetic clusters (K =4) as inferred from the STRUCTURE analyses based on the average group membership probability for that locality. Ploidy level overlaid indicates a latitudinal ploidy gradient with diploids predominant in the Northern Drakensberg and tetraploids in the Eastern Cape Drakensberg with one locality in the North, Platberg, as an outlier. **C)** A phylogenetic network analyses NeighborNet tree using Ordinary Least Squares variance. The different colours correspond to population assignment and ploidy levels from Fig. 18B, except for Platberg individuals which are marked in light blue.

The Discriminant analysis of Principal Components (DAPC) shows *R. baurii* var. *baurii* as genetically distinct from *R. baurii* var. *confecta* and *R. baurii* var. *platypetala* (Fig. 19A). *Rhodohypoxis baurii* var. *confecta* and *R. baurii* var. *platypetala* are genetically distinct but overlap somewhat. The DAPC grouped by ploidy levels shows all three ploidy levels (diploid, triploid and tetraploid) as genetically distinct with no overlap (Fig. 19B). The DAPC grouped by populations show patterns largely mirroring geographic sampling, with all the "on the main Berg" *R. baurii* var. *baurii* individuals occurring in the Eastern Cape Drakensberg (Vrederus, Naudes Nek, Tenahead lodge) grouping together, and all remaining populations (Sunset Farm, Sani Top, Sethlabethebe and Sentinel) grouping together. Two populations present as outliers, Platberg and Hebron Farm, which is consistent with the other genetic analyses (Fig. 19C).

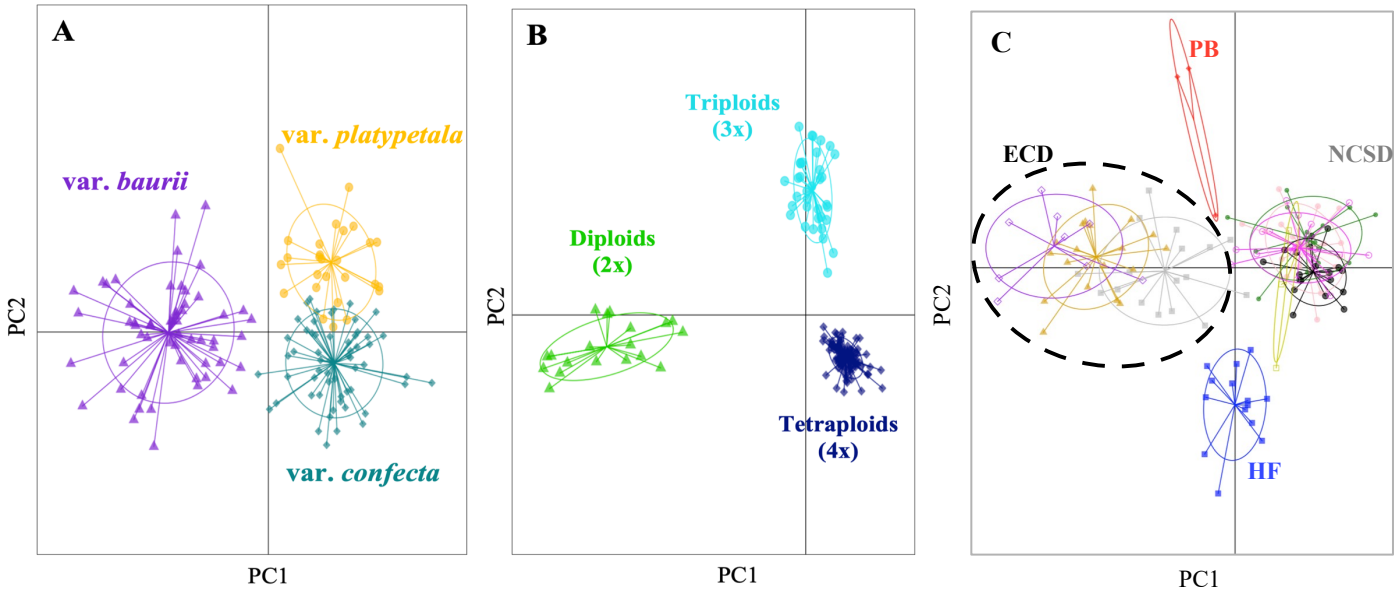

Figure 19. DAPC based on Bruvo genetic distance matrix grouped by **A**) taxonomic varieties with *R. baurii* var. *baurii* (purple triangles), *R. baurii* var. *platypetala* (yellow circles) and *R. baurii* var. *confecta* (blue diamonds), **B**) grouped by ploidy levels with diploids (2×, green triangles), triploids (3×, light blue circles) and tetraploids (4×) shown in navy diamonds and **C**) grouped by populations with each colour representing a different population, showing the Eastern Cape Drakensberg (ECD) populations grouping together on the left of PC1 (encircled by a black dashed ellipse), and the Northern, Central and Southern Drakensberg (NCSD) populations grouping together on the right of PC1 (encircled by a grey dotted ellipse). Two populations present as outliers, Platberg (PB) in red and Hebron Farm (HF) in blue. Points represent individuals and ellipses show groups identified by DAPC.

Reproductive strategies

A total of 231 crosses were performed, of which 113 crosses produced a total of 1504 seeds. All varieties and ploidy levels are capable of intra-varietal and inter-varietal inter-breeding,

indicating no postzygotic isolation between the varieties and ploidy levels, and are all capable of self-pollination to some extent. Self-pollination rates (both hand self-pollination and natural self-pollination) did not differ between the varieties with all varieties selfing at relatively low levels (~25–37% selfed individuals set seed). No varieties or ploidy levels showed apomictic breeding, as none of the emasculated individuals set seed.

Seed sets for both inter-varietal and intra-varietal crosses differed among varieties (Table 5). The intra-varietal *R. baurii* var. *baurii* crosses yielded the lowest seed set (mean seeds produced = 9.7 ± 0.432), while *R. baurii* var. *confecta* yielded the highest seed set (mean seeds produced = 21.13 ± 0.428) (Table 5). Inter-varietal crosses involving *Rhodohypoxis baurii* var. *confecta* as the maternal individual resulted in the highest seed set of all crosses (Table 5). Both intra-varietal and inter-varietal *R. baurii* var. *baurii* crosses (with *R. baurii* var. *baurii* as the maternal individual) yielded the lowest seed sets (mean seeds = 5.5 – 9.1). *Rhodohypoxis baurii* var. *platypetala* intra-varietal and inter-varietal crosses yielded similar seed sets (Table 5).

Likewise, seed sets for both inter-ploidy and intra-ploidy crosses varied greatly (Table 5). The intra-ploidy diploid-diploid crosses yielded the highest seed set (mean seeds = 12.62 ± 0.23) and triploid-triploid crosses produced the lowest seed set (mean seeds = 6 ± 0.82). However, the number of triploid individuals included in the reproductive study is low, with only two out of 12 intra-ploidy triploid crosses producing seeds (Table 5). The inter-ploidy diploid-tetraploid cross produced significantly more seeds than the other inter-ploidy crosses (mean seeds = 18.91 ± 0.021, P <0.001), and the inter-ploidy diploid-triploid crosses produced significantly less seeds (mean seeds = 5 ± 0.52, P = 0.049). Inter-ploidy crosses with triploids as the maternal individual produced lower seed sets (mean seeds = 2 ± 0.90 – 8 ± 0.83) than with triploid individuals as the paternal pollen donor (mean seeds = 5 ± 0.52 – 27 ± 0.76) (Table 5). Inter-ploidy crosses with triploids as the maternal individual produced the lowest seed set on average compared to other inter-ploidy crosses (Table 5); however, these low triploid seed sets are not significantly lower than other inter-ploidy crosses, likely due to the small cross sample size of only nine inter-ploidy crosses performed with triploid individuals.

Table 5. Differences in seed set between inter-varietal and intra-varietal crosses as well as between like ploidy and unlike ploidy crosses as evaluated by GLMMs indicating gametic incompatibilities or zygote mortality. c = *Rhodohypoxis baurii* var. *confecta,* b = *Rhodohypoxis baurii* var. *baurii,* p = *Rhodohypoxis baurii* var. *platypetala,* d = diploid, t = triploid, q= tetraploid, N seeds= total number of seeds produced from cross type, Crosses = number of successful crosses performed for cross type. Mean = mean seeds produced / successful cross, SE = Standard Error.

Intra-varietal					**Inter-varietal**					**Model outputs**		
Variety	N seeds	Crosses	Mean	SE	Variety	N seeds	Crosses	Mean	SE	Estimate	z-value	P-value
c x c	169	8	21.13	0.43	c x b	292	17	17.18	0.47	0.48	1.02	0.31
					c x p	300	22	13.63	0.31	0.23	0.75	0.45
b x b	97	10	9.7	0.43	b x c	110	15	7.3	0.0033	-0.39	-6.60	<0.001
					b x p	71	9	7.9	0.0033	-0.021	-6.21	<0.001
p x p	134	9	14.9	0.50	p x c	239	17	14.1	0.56	0.14	0.26	0.80
					p x b	92	6	15.33	0.55	0.45	0.83	0.41
Intra-ploidy					**Inter-ploidy**					**Model outputs**		
Ploidy	N seeds	Crosses	Mean	SE	Ploidy	N seeds	Crosses	Mean	SE	Estimate	z-value	P-value
d x d	820	65	12.62	0.23	d x t	25	5	5	0.52	-1.03	-1.97	0.049
					d x q	416	22	18.91	0.21	1.04	4.95	<0.001
t x t	12	2	6	0.82	t x q	4	2	2	0.90	-1.44	-1.62	0.11
					t x d	8	1	8	0.83	-0.15	-0.15	0.89
q x q	154	16	9.63	0.26	q x t	27	1	27	0.76	1.16	1.54	0.12
					q x d	152	15	10.13	0.43	-0.14	-0.51	0.61

Seeds derived from self-pollination (both hand self-pollination and natural self-pollination) did not differ in germination success between the varieties with all varieties showing relatively low seed viability (proportion germinated = 0.21 ± 0.11 – 0.38 ± 0.13). Germination of seeds resulting from inter-varietal crosses on average were higher than that of intra-varietal seeds. Germination success of inter-varietal seeds was higher when mothered by *R. baurii* var. *baurii* than for seeds that developed from *R. baurii* var. *baurii* intra-varietal crosses (Table 6). Inter-varietal crosses with *R. baurii* var. *confecta* as the maternal individual and *R. baurii* var. *baurii* as the paternal donor resulted in the highest inter-varietal germination success (proportion germinated = 0.741 ± 0.21, $P = 0.042$) and resulted in similar germination success to *R. baurii* var. *confecta* intra-varietal crosses (proportion germinated = 0.754 ± 0.25) and higher germination success than *R. baurii* var. *baurii* intravarietal crosses (prop. germinated = 0.456 ± 0.16) (Table 6). *Rhodohypoxis baurii* var. *baurii* resulted in the lowest intra-varietal germination success with less than half the seed set showing viability. Inter-varietal seeds from *R. baurii* var. *confecta* as the maternal individual and *R. baurii* var. *platypetala* as the paternal donor resulted in significantly lower germination success (proportion germinated = 0.411 ± 0.20, $P = 0.0052$) compared to other inter-varietal crosses (Table 6).

Intra-ploidy seed germination success varied widely. Diploid-diploid crosses showed the highest germination rate (prop. germinated = 0.621 ± 0.16) and triploid-triploid crosses showed the lowest germination rate, with only 1 seed out of the 12 produced germinating (prop. germinated = 0.083; Table 6). Germination success of the different inter-ploidy crosses did not differ significantly to one another (Table 6), however, the sample sizes of inter-ploidy cross type varied considerably and triploid cross data were very limited due to the few triploid crosses performed and low seed set from triploid crosses.

Crosses that yielded larger seed sets had greater germination success. Therefore, the number of seeds produced from crosses was correlated with the germination success (seed viability). Overall, an increase in seed set, irrespective of cross type, resulted in higher germination success and higher percentage of seed viability ($df = 74$, $R^2 = 0.6433$, $P < 0.001$).

Table 6. Differences in germination success between seeds from intra-varietal and inter-varietal crosses as well as between like ploidy and unlike ploidy crosses as evaluated by GLMMs indicating gametic incompatibilities or zygote mortality. c = *Rhodohypoxis baurii* var. *confecta,* b = *Rhodohypoxis baurii* var. *baurii,* p = *Rhodohypoxis baurii* var. *platypetala,* d = diploid, t = triploid, q= tetraploid; Planted = total number of seeds planted, Germ = the total number of seeds that germinated (viable seeds), mean prop = mean. proportion of seeds that germinated.

Intra-varietal					**Inter-varietal**					**Model outputs**		
Variety	Planted	Germ.	Mean prop.	SE	Variety	Planted	Germ.	Mean prop.	SE	Estimate	z-value	P-value
c x c	133	96	0.754	0.19	c x b	232	172	0.741	0.21	-0.047	-0.25	0.042
					c x p	248	102	0.411	0.20	-0.56	-2.79	0.0052
b x b	76	35	0.456	0.16	b x c	96	65	0.677	0.30	0.55	1.81	0.071
					b x p	56	35	0.625	0.35	0.51	1.47	0.14
p x p	79	51	0.646	0.39	p x c	202	115	0.569	0.18	-0.27	-1.53	0.13
					p x b	67	33	0.47	0.47	-0.72	-1.54	0.12
Intra-ploidy					**Inter-ploidy**					**Model outputs**		
Ploidy	Planted	Germ.	Mean prop.	SE	Ploidy	Planted	Germ.	Mean prop.	SE	Estimate	z-value	P-value
d x d	700	435	0.621	0.16	d x t	21	11	0.524	0.47	-0.28	-0.59	0.55
					d x q	303	154	0.508	0.16	-0.23	1.39	0.16
t x t	12	1	0.083	2.64	t x q	9	8	0.899	2.63	-2.3	0.01	0.91
					t x d	7	0	0	<0.001	-0.0028	0.01	0.90
q x q	116	54	0.466	0.24	q x t	20	16	0.800	0.47	0.39	0.82	0.41
					q x d	115	62	0.549	0.34	0.034	0.098	0.92

Pollen viability was assessed using Alexander's stain and compared between varieties and ploidies (Fig. 20). Mean pollen viability did not differ significantly between the three varieties *(*df = 62, *F* = 0.60, *P* = 0.53*)* (Fig. 20a), and all three varieties showed variable levels of pollen viability with very low and very high pollen viability among different individuals. However, pollen viability differed greatly between ploidy levels. Triploids exhibited significantly lower pollen viability than diploids and tetraploids (df = 62, *F* = 15.79, *P* = 0.00001) (Fig. 20b). Pollen viability did not differ between diploids and tetraploids (df = 62, *F* = 0.60, *P* = 0.31) (Fig. 20b).

It is common for triploid individuals to exhibit deformed pollen grains with low viability, therefore pollen grains of *R. baurii* individuals with different ploidies were imaged using a scanning electron microscope (SEM). Only two individuals per ploidy were imaged to assess if there were morphological differences between pollen grains. Diploid and tetraploid pollen grains exhibited normal morphology (Fig. 21a & 21c) however, the triploid pollen was deformed and did not exhibit typical morphology (Fig. 21b). Furthermore, tetraploid pollen grains appeared significantly larger than diploid pollen grains (Fig. 21a & 21c), but more sampling and measurements would need to be conducted to confirm any trends or differences.

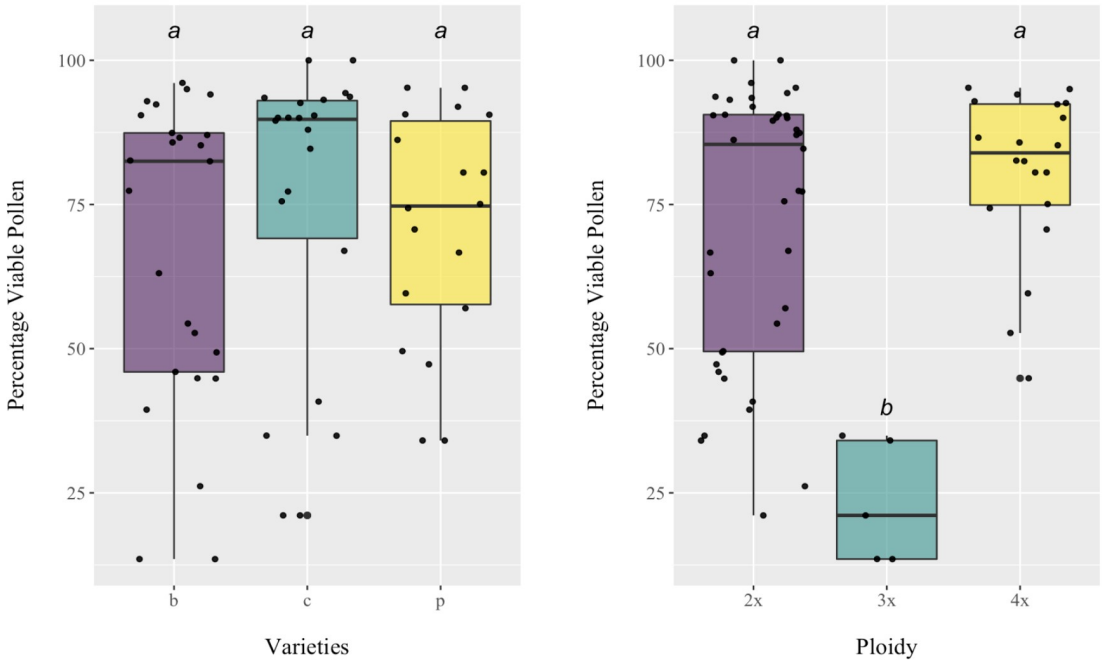

Figure 20. Comparison of pollen viability between (A) *Rhodohypoxis baurii* varieties (*R. baurii* var. *baurii, R. baurii* var. *confecta* and *R. baurii* var. *platypetala)* and (B) different ploidy levels (diploid (2×), triploid (3×) and tetraploid (4×)) among *Rhodohypoxis baurii* varieties.

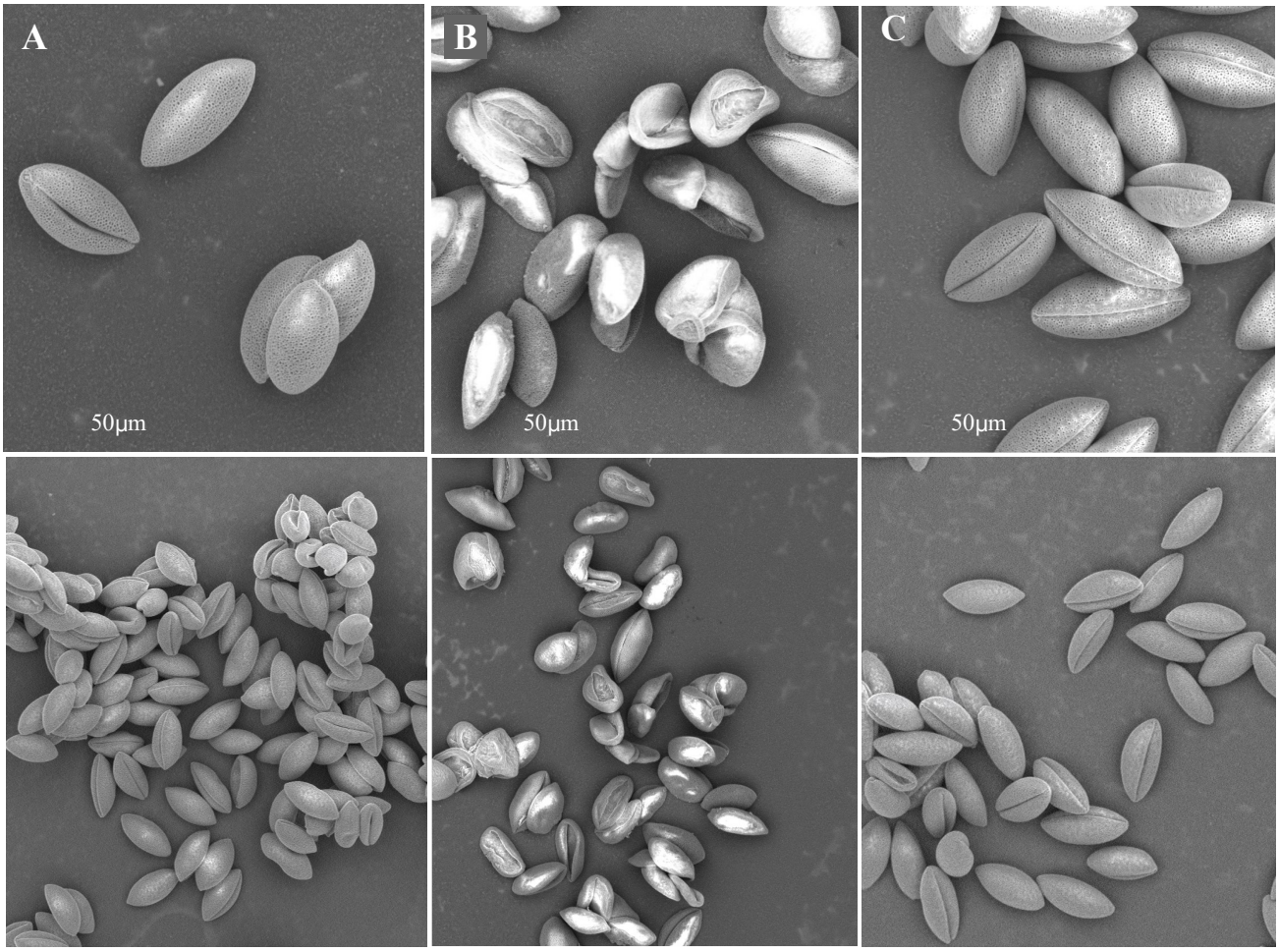

Figure 21. Desktop Scanning Electron Microscope (SEM) images of *R. baurii* pollen grains. Note the deformed triploid pollen, and larger tetraploid pollen. A: diploid (2×) *Rhodohypoxis baurii* var. *confecta* pollen, B: triploid (3×) *Rhodohypoxis baurii* var. *platypetala* pollen and C: tetraploid (4×) *Rhodohypoxis baurii* var. *baurii* pollen. All scale bars = 50um.

Clonal asexual reproduction was evaluated by checking for clonal root connections in the pots of individuals house in the greenhouse, and by using microsatellite markers to evaluate if individuals had identical genetic profiles. Twenty percent of *R. baurii* var. *confecta* individuals shared root connections with other individuals within the same pots and 28.57% were genetically clonal to one another in the same pots in the greenhouse as well as in natural populations (Table 7). Of the *R. baurii* var. *baurii* individuals, 36.4% shared root connections within the same pot and 11.54% were genetically clonal. Lastly, *Rhodohypoxis baurii* var. *platypetala* showed significantly higher clonal reproduction than *R. baurii* var. *baurii* and *R. baurii* var. *confecta* (df = 155*, F = 2.99, P* < 0.0001) with 40% of *R. baurii* var. *platypetala* individuals having shared clonal root connections, and 77.08% of individuals in the genetic dataset having near identical genotypes (Table 7). *Rhodohypoxis baurii* var. *platypetala*

showed the highest instances of clonal reproduction, but also contains multiple ploidy levels and has the highest number of triploid individuals. Therefore, clonality was compared between ploidy levels. Diploids and tetraploids showed similar degrees of clonal reproduction (df = 155, $F = 1.54$, $P = 0.88$) with 26.42% of diploids sharing root connections and 31.03% of tetraploid individuals sharing root connections respectively. Furthermore, 33.89% of diploid individuals and 32.22% of tetraploids were genetically clonal, therefore showing similar levels of clonality (df = 155, $F = 1.69$, $P = 0.96$; Table 6). Based on the microsatellite data, triploids showed significantly higher rates of clonal reproduction than diploids (df = 155, $F = 19.77$, $P = 0.023$) and tetraploids (df = 155, $F = 12.22$, $P = 0.036$; Table 7).

Table 7. Differences in clonal reproduction between *Rhodohypoxis baurii* varieties and ploidy levels for (A) root connections observed in pots housed in the greenhouse and (B) clonality by genotype data with individuals exhibiting identical genotypes (as identified by GenoDive) noted as clones. c = *Rhodohypoxis baurii* var. *confecta,* b = *Rhodohypoxis baurii* var. *baurii,* p = *Rhodohypoxis baurii* var. *platypetala,* 2× = diploid, 3× = triploid, 4× = tetraploid.

	Varieties of *Rhodohypoxis baurii*					Ploidy-levels within *Rhodohypoxis baurii*				
	Variety	No. clones/ total	Percent clones	Comp.	P-value	Ploidylevel	No. clones	Percent clones	Comp.	P-value
A: Root connections	baurii	12/33	36.36	c-b	0.350	2×	14/53	26.42	3×-2×	0.039
	confecta	6/30	20.00	c-p	0.255	3×	7/11	63.63	4×-2×	0.88
	platypetala	12/30	40.00	p-b	0.977	4×	9/29	31.03	4×-3×	0.12
B: Msat data	baurii	9/78	11.54	c-b	0.040	2×	20/59	33.89	3×-2×	0.023
	confecta	10/35	28.57	c-p	<0.001	3×	6/11	54.54	4×-2×	0.96
	platypetala	37/48	77.08	p-b	<0.001	4×	29/90	32.22	4×-3×	0.036

Pollinator observations across R. baurii *varieties*

Pollinator observations were conducted at seven sites (Table 8) to assess what was potentially serving as a pollen transfer vector between individuals. In large populations of *R. baurii* var. *baurii,* a faint sweet scent was apparent, and a faint sweet musky scent was present at all

times in populations of *R. baurii* var. *confecta*. Flies, bees, ants, wasps, and beetles were observed visiting *R. baurii* flowers of all varieties (Table 8, Fig. 22A), but very few interactions were likely to lead to pollination. Flies were observed at higher altitude populations such as Golden Gate and Sentinel (2060 m a.s.l. and 2600 m a.s.l. respectively) and other insect visitors such as bees and beetles were found at lower altitude populations such as Karkloof (1768 m a.s.l.). Pollinator observations did not identify any specific pollinator, however herbivory of the tepals and anthers of the plants by beetles was frequently evident (Fig. 22B). For instance, many observed flowers were missing anthers entirely (Fig. 22B1) and degrees of herbivory were noticeable on the plants (Fig. 22B). Certain insects caught in the field, such as ants and flies, were found to be carrying *R. baurii* pollen in small quantities (6 – 14 pollen grains per individual) (Fig. 23E – G; Table 8). Other insects such as the Braconid wasp (Fig. 23D) were found carrying larger amounts of *R. baurii* pollen (ca. 40 pollen grains). All insects that were carrying *R. baurii* pollen were also found to be carrying pollen from other plant species but in higher quantities (Table 8).

Table 8. Summary of insect activity of species observed on inflorescences of *Rhodohypoxis baurii* at seven sites throughout the Drakensberg during the November 2021– January 2022 flowering season. Insects caught were analysed for pollen load and imaged for identification as seen in Figure 23 and noted in the table below. Pollinator observations were conducted at seven sites: Golden Gate National Park, Sentinel, Langalibalele Pass, Karkloof Nature Reserve, Minerva Nature Reserve, Highmoor Reserve and Vrederus Trout Farm.

Variety	Population & Altitude (m a.s.l)	Insect	Visitation type	Flower colour	Scent present	Insect caught	# viable *Rhodohypoxis* pollen grains	# viable other pollen grains
R. baurii var. *platypetala*	Golden Gate 2060 m a.s.l	Bombyliidae (Bee fly)	± 20s Brief, moving across several flowers Evidence of herbivory, tepals and anthers eaten.	White	Faint sweet scent	N	NA	NA
R. baurii var. *platypetala*	Golden Gate 2060 m a.s.l	*Daspletis placodes* (Robber fly)	± 30s Brief, moving across several flowers	White	Faint sweet scent	Y (Fig. 23G)	8	18
R. baurii var. *platypetala*	Golden Gate 2060 m a.s.l	Anthomyiidae (Root-maggot fly)	± 5-10s Brief, landing and leaving immediately	White	Faint sweet scent	N	NA	
R. baurii var. *platypetala*	Golden Gate 2060 m a.s.l	Drosophiliidae (Vinegar fly)	± 5-10s Brief, landing and leaving immediately	White	Faint sweet scent	Y (Fig. 23E)	14	26
R. baurii var. *platypetala*	Golden Gate 2060 m a.s.l	*Linepithema* sp. (Argentine ant)	± 30s Climbing around flower, going in and under tepals onto anthers in flower centre.	White	Faint sweet scent	Y (Fig. 23F)	6	30
R. baurii var. *platypetala*	Golden Gate 2060 m a.s.l	*Messor* sp. (Harvester ant)	± 45s Climbing around flower, going in and under tepals onto anthers in flower centre. Evidence of herbivory, tepals and anthers eaten.	Pink and white mix	Faint sweet scent	N	NA	NA
R. baurii var. *platypetala*	Golden Gate 2060 m a.s.l	*Messor* sp. (Harvester ant)	± 45s Climbing around flower, going in and under tepals onto anthers in flower centre.	Pink and white mix	Faint sweet scent	N	NA	NA
R. baurii var. *platypetala*	Golden Gate 2060 m a.s.l	*Messor* sp. (Harvester ant)	± 30s Climbing around flower, going in and under tepals onto anthers in flower centre.	White	Faint sweet scent	N	NA	NA
R. baurii var. *platypetala*	Golden Gate 2060 m a.s.l	*Linepithema* sp. (Argentine ant)	± 45s Climbing around flower, going in and under tepals onto anthers in flower centre.	Pink	Faint sweet scent	N	NA	NA
R. baurii var. *platypetala*	Golden Gate 2060 m a.s.l	Drosophiliidae (Vinegar fly)	±20s Resting on tepals and walking on tepals	White	Faint sweet scent	N	NA	NA
R. baurii var. *confecta*	Sentinel 2600 m a.s.l	Anthomyiidae	± 30s Brief, bouncing across several flowers	Pink and white mix	Sweet/ musky scent	Y (Fig. 23A)	9	33

		(Root-maggot fly) (*Anthomyia ochripes)*						

R. baurii var. *confecta*	Sentinel 2600 m a.s.l	Braconidae (Braconid wasps)	± 60s Resting on tepals and walking on tepals	Pink and white mix	Sweet/ musky scent	Y (Fig. 23D)	40	80
R. baurii var. *confecta*	Sentinel 2600 m a.s.l	Anthomyiidae (Root-maggot fly)	± 30s Brief, bouncing across several flowers	Pink and white mix	Sweet/ musky scent	Y (Fig. 23B)	0	0
R. baurii var. *confecta*	Sentinel 2600 m a.s.l	Anthomyiidae (Root-maggot fly)	± 30s Brief, bouncing across several flowers	Pink and white mix	Sweet/ musky scent	Y (Fig. 23C)	8	30
R. baurii var. *confecta*	Sentinel 2600 m a.s.l	Anthomyiidae (Root-maggot fly)	±90s Resting on tepals and walking on tepals	Pink and white mix	Sweet/ musky scent	Y (Fig. 23H)	0	25
R. baurii var. *platypetala*	Langalibalele pass	*Clania* sp. (Monkey beetle)	Several minutes Pulling tepals apart, trying to get into the centre of flower towards the anthers	White	No scent	N	NA	NA
R. baurii var. *platypetala*	Karkloof 1768 m a.s.l.	Chrysomelidae (Green leaf beetle)	Remained on tepal for entire observation period. Sitting under top whorl of tepals, eating tepals.	White	No scent	N	NA	NA
R. baurii var. *platypetala*	Karkloof 1768 m a.s.l.	Halictidae (Flower bee)	Remained on tepal for entire observation period. Sitting under the tepal	White	No scent	N	NA	NA
R. baurii var. *platypetala*	Minerva 1500 m a.s.l.	NA	No insects observed	White population	No scent	N	NA	NA
R. baurii var. *platypetala*	Highmoore 1980 m a.s.l.	NA	No insects observed	White population	No scent	N	NA	NA
R. baurii var. *baurii*	Vrederus 1950 m a.s.l.	NA – evidence of herbivory	No insects observed but clear herbivory of tepals	Dark pink/red	Stronger sweet scent	N	NA	NA

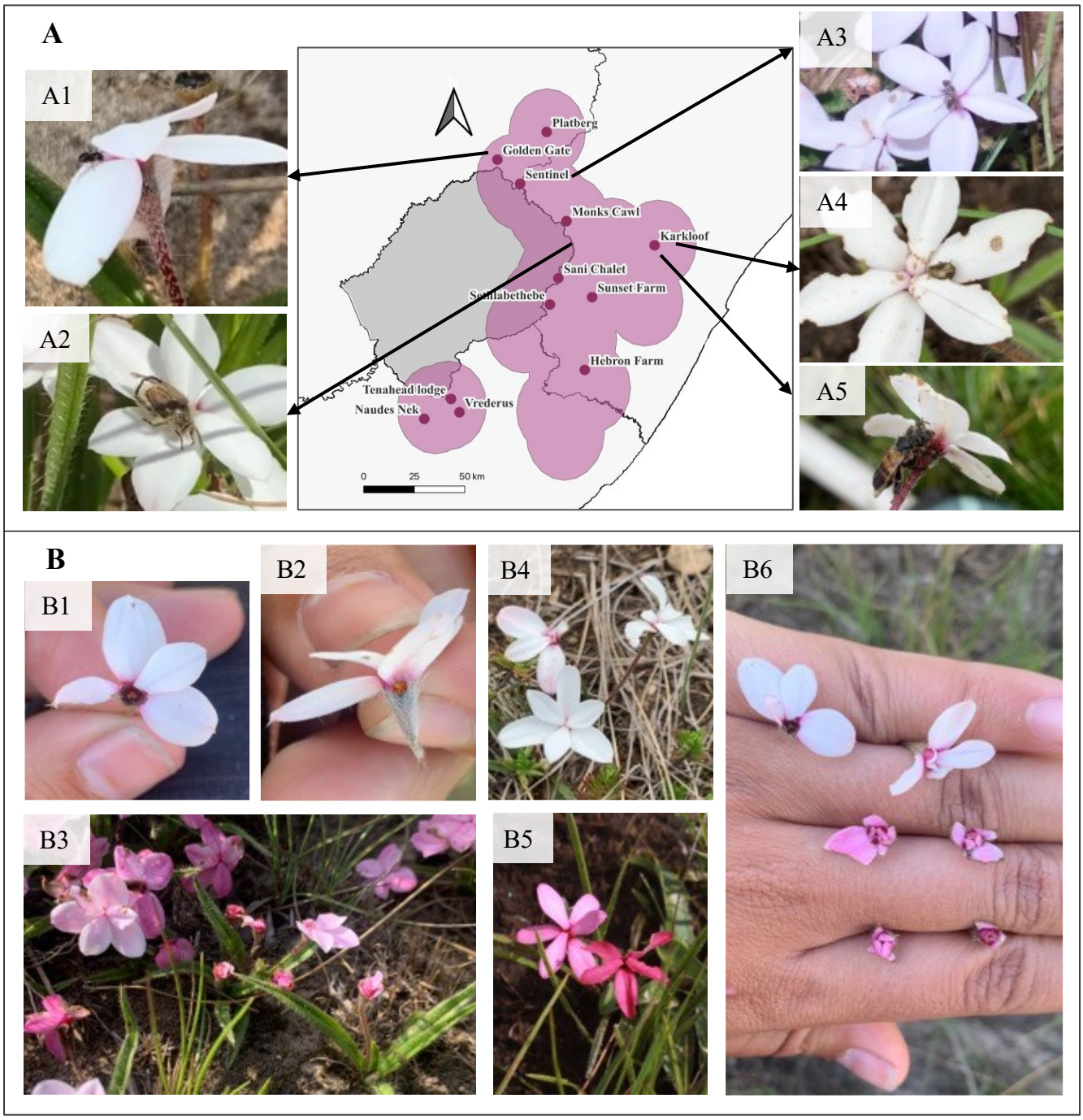

Figure 22. (**A)** Insect visitors of *Rhodohypoxis baurii* flowers at several different populations during pollinator observations during the November 2021– January 2022 flowering season. A1) Family Formicidae (*Messor* sp.) on a *R. baurii* var. *platypetala* tepal at Golden Gate National Park, A2) Monkey beetle (*Clania* sp.) on a *R. baurii* var. *platypetala* tepal at Langalibalele Pass, A3) Fly on a *R. baurii* var. *confecta* tepal at Sentinel Car Park, A4) Green leaf beetle (Family Chrysomelidae) on a *R. baurii* var. *platypetala* tepal at Karkloof, A5) Flower bee (Family Halictidae) on a *R. baurii* var. *platypetala* tepal at Karkloof. (**B)** Evidence of herbivory on the tepals and anthers of *Rhodohypoxis baurii* varieties. B1-B4) Evidence of herbivory on tepals, anthers and subsequently pollen at Golden Gate National Park, B5) Tepal herbivory of *R. baurii* var. *baurii* at Tenahead Lodge. B6) Heavy herbivory on multiple *R. baurii* var. *confecta* flowers at Sentinel.

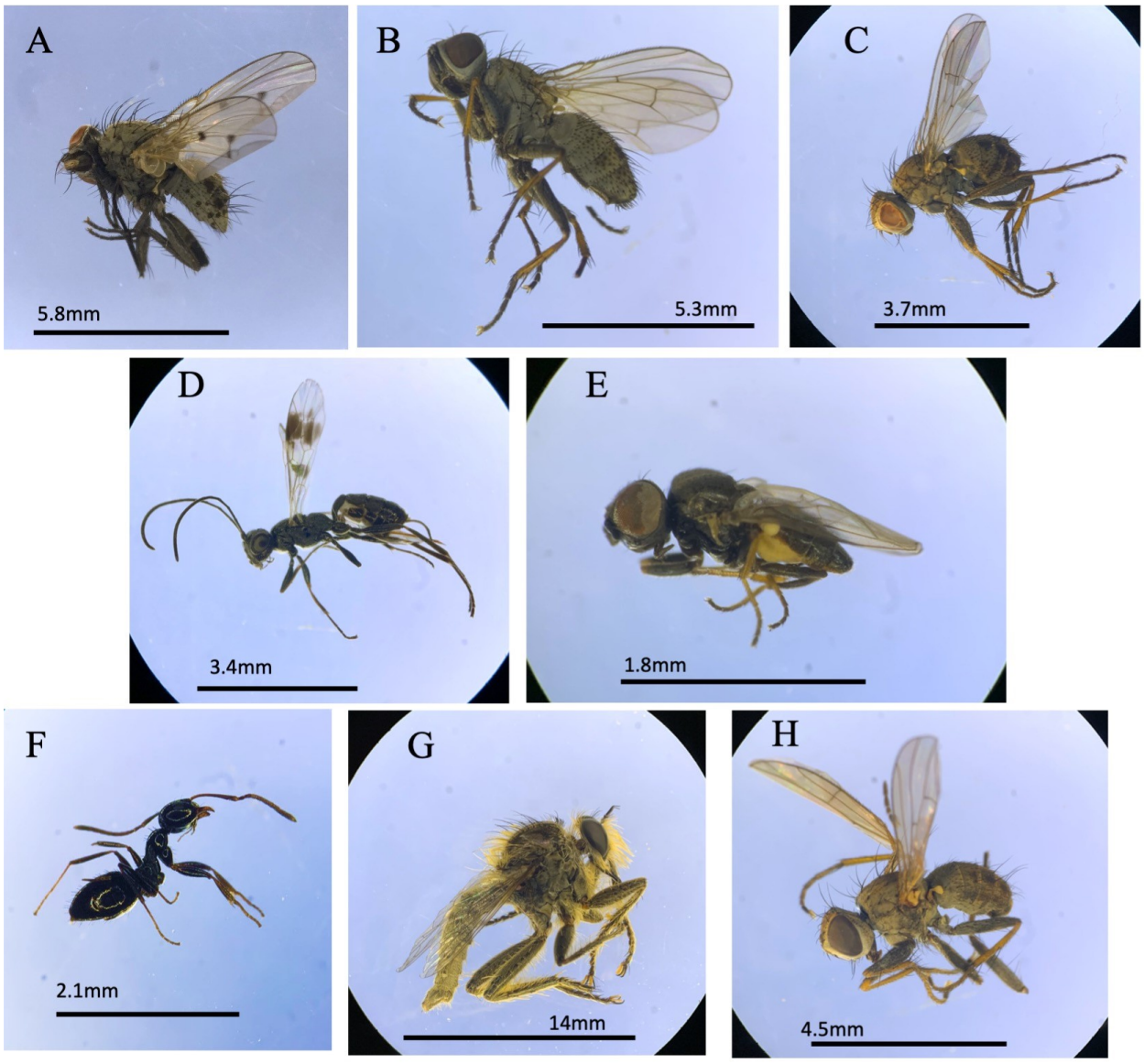

Figure 23. Frequent insect visitors caught whilst visiting *Rhodohypoxis baurii* flowers at Sentinel (A-E) and Golden Gate Highlands national Park (F-H) during pollinator observations during the peak of the flowering season from November 2021 – January 2022. A) Anthomyiidae - *Anthomyia ochripes* (Root-maggot fly), B) Anthomyiidae (Root-maggot fly), C) Anthomyiidae (Root-maggot fly), D) Braconidae family (Braconid wasps), E) Drosophiliidae family (Vinegar fly), F) *Linepithema* (Argentine ant), G) Robber fly in genus *Daspletis,* H) Anthomyiidae (Root-maggot fly).

CHAPTER 4: DISCUSSION

The purpose of this study was to assess the implications of chromosome duplication (polyploidy) on morphology, genetic differentiation and reproductive strategy amongst varieties of *Rhodohypoxis baurii,* and to better distinguish the three varieties. Ploidy levels varied across the varieties and populations with *R. baurii* var. *platypetala* showing the highest cytotype diversity with diploid, triploid and tetraploid individuals found across multiple populations. *Rhodohypoxis baurii* var. *baurii* contained both diploids and tetraploids, whereas *Rhodohypoxis baurii* var. *confecta* consisted only of diploid individuals (with the exception of a few isolated polyploid hybrids found at Platberg). Golden Gate and Sentinel populations found in the Northern Drakensberg consisted exclusively of diploid individuals whereas Tenahead Lodge, Vrederus and Naudes Nek Eastern Cape Drakensberg populations consisted entirely of tetraploids. All the Central and Southern Drakensberg populations consisted of a mix of diploid, triploid and tetraploid individuals in each population. The Platberg population presented as an outlier as it is located in the Northern Drakensberg close to Sentinel and Golden Gate but does not consist exclusively of diploid individuals, instead it contains diploid, triploid and tetraploid individuals.

How does polyploidy influence morphology in R. baurii *varieties?*

Interestingly, it was found that many morphological traits did not differ significantly between *R. baurii* diploid and polyploid (triploid and tetraploid, inclusive) individuals, with only a few traits such as flower size and anther size differing. However, polyploids exhibited greater variation in certain traits, such as leaf width and leaf length. Polyploidization often leads to increased morphological variation due to the increased genetic variation (McCarthy *et al.*, 2016; Soltis *et al.*, 2014). Morphological changes and increased morphological variation as well as increased phenotypic plasticity may provide polyploids with an adaptive advantage in stressful environments (Burton & Husband, 2000; Husband et al., 2013; Maherali et al., 2009; Thompson et al., 2015). However, polyploid *R. baurii* individuals weren't always found in more stressful environments as *R. baurii* var. *confecta* consists entirely of diploid individuals and populations of *R. baurii* var. *confecta* were often found at higher altitudes (2100–3000 m a.s.l).

Polyploidization not only enables morphological variability due to increased genetic variation, but often leads to changes in trait sizes (McCarthy *et al.*, 2016; Soltis *et al.*, 2014).

Out of the 20 morphological traits measured in *R. baurii*, only four traits differed significantly between ploidy levels across all varieties. All tetraploid *R. baurii* var. *platypetala* individuals and tetraploid *R. baurii* var. *baurii* individuals from the Southern Drakensberg and away from the main Drakensberg populations have longer outer and inner tepals (larger flowers) and have larger anthers (longer and wider anthers) compared to diploid individuals. The Eastern Cape Drakensberg *R. baurii* var. *baurii* populations consists exclusively of tetraploid individuals and are morphologically distinct from all other populations and varieties. However, the correlation between the morphological differences and polyploidy seen in the Eastern Cape Drakensberg populations do not coincide with the general polyploid trends that we have observed thus far, as the Eastern Cape Drakensberg populations have smaller tepals, shorter peduncles and thinner leaves with little morphological variation. Eastern Cape Drakensberg *R. baurii* var. *baurii* populations are very unique as they are geographically isolated from all other *R. baurii* varieties, exhibit unique genotypes, occur at high altitudes and occur in a region that receives higher amounts of rainfall and snow fall (i.e. ecologically different) than the Southern and Northern Drakensberg populations. Therefore, the Eastern Cape Drakensberg *R. baurii* populations are not good representatives to understand the effects of polyploidy on morphology in *R. baurii* and are addressed separately "Taxonomic Implications".

Polyploid individuals often display larger morphological traits (as seen in polyploid *R. baurii* var. *platypetala* and *R. baurii* var. *baurii* excluding the Eastern Cape Drakensberg populations), due to cell enlargement, i.e. the gigas effect (Müntzing, 1936; Otto & Whitton, 2000; Stebbins, 1971). Polyploids that are morphologically larger in habit and have larger reproductive traits (Bretagnolle & Lumaret, 1995; Garbutt & Bazzaz, 1983) have been shown to exhibit increased competitiveness (Burton & Husband, 2000; Hülber et al., 2011; Maceira et al., 1993; Schlaepfer et al., 2010). Increased competitiveness of *R. baurii* polyploid individuals is likely occurring. Polyploid *R. baurii* individuals generally have larger floral and reproductive traits and are capable of reproducing using multiple different strategies (i.e. self-pollination, intra- and inter-varietal crossing and clonal reproduction). Furthermore, polyploid *R. baurii* individuals and populations have the widest known current geographic distribution occurring in the Northern (i.e. Platberg), Central, Southern and Eastern Cape Drakensberg regions as well as occurring in populations away from the main Drakensberg (e.g. Hebron Farm and Karkloof). The distribution of diploid *R. baurii* individuals and populations are restricted to the Northern, Central and Southern Drakensberg regions, and are

entirely missing from the Eastern Cape Drakensberg. Furthermore, the number of diploid individuals present in populations decreases drastically in the Central and Southern Drakensberg, where populations contain multiple ploidy-levels and are polyploid dominated, compared to the Northern Drakensberg populations which consists of diploids only. Furthermore, populations with mixed ploidies such as Sunset Farm show lower numbers of diploids compared to polyploids, indicating that polyploid *R. baurii* individuals may have increased competitiveness compared to diploid *R. baurii*. Therefore, there is evidence that polyploidy in *R. baurii* individuals, together with a shift towards larger floral and reproductive traits, has resulted in increased competitiveness in *R. baurii* polyploids. However, competitiveness of the varieties has not been directly tested and further investigations (using common garden experiments) would be needed to confirm the above theories.

Polyploid *R. baurii* var. *platypetala* individuals from Karkloof exhibited larger tepals and increased pollinator activities were observed at this population compared to *R. baurii* var. *platypetala* populations that had smaller flowers, such as Minerva Private Reserve (found away from the main Drakensberg in KZN) and Highmoor Nature Reserve. Changes in reproductive traits and floral morphology, such as larger tepal lengths, as seen in some *R. baurii* varieties can potentially lead to an increase in pollinator visitation frequencies (Kennedy et al., 2006), leading to shifts in pollinator interactions (Gross & Schiestl, 2015; McCarthy *et al.*, 2015; Segraves & Thompson, 1999). However, there are many other variables that could be resulting in differences in insect visitation between the populations. The localities of the populations differ in humidity, temperature and elevation which could potentially be influencing both pollinator abundance and visitation. Vegetation of the immediate surroundings for all populations is similar but the mosaic of landscapes surrounding the conserved sites where the populations occur differs. For example, Karkloof population is surrounded by pine plantations, whereas as Sentinel is surrounding by natural alpine-montane grasslands. However, the populations that exhibited the highest frequency of insect visitation were not polyploid populations, but diploid *R. baurii* var. *confecta* populations that contained higher numbers of pink flowers (such as Sentinel). It was observed that flowers with pink tepals had significantly higher frequency of insect herbivory on tepals and anthers, likely resulting in coincidental pollen transfer. Furthermore, the types of insects visiting the larger white flowered polyploid *R. baurii* var. *platypetala* at Karkloof differed to those observed visiting smaller *R. baurii* var. *confecta* pink flowers but populations that were

geographically close (such as Sentinel and Golden Gate) exhibited similar kinds of insect visitors, irrespective of flower colour.

The observed insect interactions noted above, and possible coincidental pollen transfers are supported by our molecular data as the diploid Sentinel and Golden Gate populations that contained different varieties with different flower colours shared high numbers of alleles indicating high gene flow between these populations. On the other hand, the mixed ploidy Karkloof population had a unique genotype, indicating little to no gene flow between the Karkloof population and other populations, but different ploidies within the Karkloof population did not have unique genotypes, indicating gene flow between different ploidy-levels within the same population. However, Karkloof population is a low altitude (ca. 1760 m a.s.l) geographically isolated populations therefore the reason for the unique genetic profile could be due to ecology and location, and not due to polyploidy. Changes in pollinator visitation may result in higher degree of assortative mating (Porturas *et al.*, 2019; Segraves & Anneberg, 2016) or may result in reproductive isolation by the loss of shared pollinators, resulting in changes in gene flow. This may have lead to reproductive isolation in polyploid *R. baurii* individuals or in the white-flowered *R. baurii* variety, or may be a mechanism by which polyploid *R. baurii* individuals are able to escape minority cytotype exclusion (MCE; Levin, 1975; McCarthy et al., 2016; Van de Peer et al., 2017).

Reproductive strategies and escaping MCE in R. baurii

Results from intra- and interploidy crosses indicate that there is a shift from sexual reproduction to asexual (clonal) reproduction in *R. baurii* polyploids. Triploid and tetraploid intra-varietal crosses, regardless of variety, show lower seed sets and germination rates than diploid intra-varietal crosses. Triploid and tetraploid *R. baurii* individuals also show higher rates of clonal reproduction than diploid individuals. *Rhodohypoxis baurii* var. *platypetala* shows the highest rate of clonal reproduction and contains the largest number of polyploid individuals. Furthermore, certain populations that contain multiple ploidy levels, such as Hebron Farm and Karkloof, show higher instances of clonal reproduction than populations that contain a single cytotype, such as Golden Gate and Sentinel. This indicates that a switch from sexual to asexual reproduction could be one mechanism by which *R. baurii* polyploids are escaping MCE. However, without a dated phylogeny of the genus, it cannot be said with certainty whether polyploids or diploids are the ancestral form of *R. baurii* or whether the differences in reproduction observed are due to a switch from sexual to asexual or vice versa

as we do not know the original ploidy and reproductive mode of the genus and species. It is important to note that clonal reproduction is occurring in natural populations, showing that the clonal reproduction observed is not due to greenhouse conditions or a shift in breeding systems in an artificial environment, but is a reproductive mechanism in natural populations. Newly establishing polyploids are likely to be in sympatry with their diploid progenitors when they first form, and crosses between different cytotypes generally lead to reduced fitness (Jackson, 1976; Levin, 1975; Ramsey & Schemske, 1998), resulting in minority cytotype disadvantages (Levin, 1975). Therefore, the ability to self-pollinate or reproduce asexually allows polyploid individuals to escape MCE, survive, and reproduce (Levin, 1975; Ramsey & Schemske, 1998; Stebbins, 1971). Asexual reproduction tended to be more common than sexual reproduction in triploid *R. baurii* individuals, and polyploid *R. baurii* individuals showed higher rates of asexual reproduction than diploids. Switching from sexual to asexual reproduction or increasing propensity for selfing can reinforce niche differentiation (Hörandl, 2006; Kao, 2008; Rausch & Morgan, 2005; Rodriguez, 1996), allowing for competition avoidance which is important for polyploid establishment and speciation (Weiss-Schneeweiss et al., 2013).

However, even though there is a shift from sexual to asexual reproduction in *R. baurii* individuals, sexual reproduction is still occurring and inter-varietal crosses yielded higher seed set and germination success than intra-varietal crosses. Higher inter-varietal reproduction indicates higher assortative mating, which contradicts the increased tendency towards asexual reproduction. Both asexual reproduction and increased assortative mating occurring independently may assist and result in niche differentiation, polyploid establishment and polyploid speciation and may both be mechanisms by which polyploid *R. baurii* individuals are evading MCE. Therefore, it is likely that different populations of *R. baurii* polyploids are exhibiting either increased asexual reproduction or increased assortative mating, but not both. For example, the Karkloof population consists exclusively of *R. baurii* var. *platypetala* and contains both diploid and tetraploid cytotypes and polyploid individuals at Karkloof show high instances of clonal reproduction. Furthermore, the Karkloof population is geographically isolated from other populations and varieties and exhibits a unique genotype, indicating low levels of inter-varietal crossing, resulting in no assortative mating. On the contrary, Sunset Farm population has diploid, triploid and tetraploid ploidy levels present but polyploid individuals show lower levels of asexual reproduction compared to Karkloof polyploids. Furthermore, gene flow is occurring between Sunset Farm *R. baurii* var. *platypetala* and Sani Chalet *R. baurii* var. *confecta* indicating a higher degree of

assortative mating. Therefore, both assortative mating and a switch to asexual reproduction may be mechanisms by which polyploid *R. baurii* individuals are able to escape MCE, with different populations exhibiting different reproductive escape mechanisms.

Any crosses among *R. baurii* varieties that involved triploid individuals yielded low seed set and germination rates, which was expected as triploid *R. baurii* individuals showed more deformed inviable pollen grains than other cytotypes. However, some normal viable triploid pollen grains were observed, and inter-ploidy triploid crosses still produced some viable seeds. This indicates that gene flow is not being completely blocked between triploid individuals and *R. baurii* triploids could be acting as triploid bridges, facilitating gene flow between ploidies. Autotriploid gametes are often not viable but can mediate and lead to tetraploid production via self-fertilisation or backcrossing with diploids (Dermen, 1931; Husband, 2000; Johnsson, 1940; Levin, 1975; Ramsey & Schemske, 1998; Yamauchi *et al.*, 2004). The resulting tetraploids generally have functional gametes and can reproduce further (Yamauchi *et al.*, 2004). Self-fertilisation and backcrossing of hybrid triploids with diploids often leads to allotetraploid formation (Ramsey & Schemske, 1998), and can result in gene flow between unlike ploidies. Therefore, triploid bridges may be another mechanism by which *R. baurii* polyploid individuals are potentially escaping MCE.

It is clear from the orientation of the sexual organs of *R. baurii* that the species is morphologically equipped for self-pollination, however, gene flow occurs between *R. baurii* varieties and ploidies. Therefore, pollen or gametic transfer via a vector must be occurring. Due to the morphology of the flower and position of the anthers inside the closed tepals, wind pollination is unlikely to be occurring. Pollinator observations were conducted at seven sites to assess what was potentially serving as a pollen transfer vector between individuals. In large populations of *R. baurii* var. *baurii,* a faint sweet scent was noticeable, and a faint sweet musky scent was present at all times in populations of *R. baurii* var. *confecta.* Flies, bees, ants, wasps, and beetles were observed visiting *R. baurii* flowers of all varieties, but very few observed interactions were likely to lead to pollination. Yet, Braconid wasps and Argentine ants were found to be carrying *R. baurii* pollen and may facilitate local allele sharing. Furthermore, herbivory of the tepals and anthers was apparent (likely by beetles) and therefore, coincidental pollen transfer is possibly occurring due to feeding behaviours. Increased pollinator activity was observed in *R. baurii* var. *platypetala* with larger flowers

(larger tepals) and increased herbivory activity (possibly leading to accidental pollen transfer) was noted in populations with higher frequency of pink flower morphs.

Increased pollinator activity due to shifts in flower morphology and colour has likely resulted in increased assortative mating in certain populations *R. baurii* as discussed previously. A shift away from sexual reproduction, together with triploid bridges and an increase in negative assortative mating (indicated by higher inter-varietal and inter-ploidy cross success), has likely allowed *R. baurii* polyploid individuals to evade MCE, perpetuate in current populations and establish in new populations.

Genetic diversity and gene flow across R. baurii *varieties*

Polyploid *R. baurii* individuals show higher observed heterozygosity than diploids and lower inbreeding than diploids. This was surprising as *R. baurii* polyploid individuals had more observed instances of selfing and asexual reproduction, which often results in lower genetic diversity (albeit usually in diploids) and, additionally, polyploid individuals often experience genetic bottlenecks upon formation (Moody et al., 1993). However, clonal reproduction does not necessarily lead to a decrease in genetic diversity, if genetic diversity of the population was already high, clonal reproduction may simply maintain genetic diversity (Bengtsson, 2003; Ellstrand & Roose, 1987; Hebert, 1987), and polyploids usually have higher genetic diversity than their diploid progenitors to start with (Moody et al., 1993; Soltis & Soltis, 2000). Therefore, due to the high genetic diversity in *R. baurii* polyploids despite the prevalence of asexual reproduction, *R. baurii* polyploidy populations must already have had high genetic diversity that is now being maintained by clonal reproduction, or other mechanisms, such as gene flow, or the presence of novel alleles could be resulting in higher genetic diversity in polyploids.

Many factors can contribute to, and result in, polyploids exhibiting higher genetic diversity relative to diploids such as the presence of novel alleles, polyphyletic origins and/or gene flow patterns. The presence of novel alleles in tetraploid populations can contribute to polyploid genetic diversity as polyploids generally have a greater number of alleles and, therefore, are more likely to experience random mutations that would result in higher observed genetic diversity (Otto & Whitton, 2000; Paquin & Adams, 1983). A larger number of polyploid species are polyphyletic and, therefore, are thought to have higher genetic diversity because polyphyletic formation integrates genetic diversity from multiple lineages

(Soltis & Soltis, 2000). Multiple polyploids originating from different lineages could contribute to genetic diversity. However, the origin of *R. baurii* polyploids cannot be determined from our data, and the high genetic diversity cannot be attributed to polyphyletic origins without further investigations as the STRUCTURE analysis indicates that multiple origins of polyploids in *R. baurii* is unlikely. Higher genetic diversity and variation could be a result of gene flow among independent polyploid origins (Glennon & Church, 2015; Soltis & Soltis, 1999). However, many biases exist in the indices used to evaluate genetic diversity and differentiation such as Bruvo's distances. Bruvo's distance assumes a stepwise mutation model (SMM) which allows genetic relationships between individuals to be analysed in higher precision using allele size differences as proxy for genetic distance. Bruvo's genetic distance is one of the recommended measures for dissimilarity analyses, especially when there is variation in ploidy in the study species (Kosman & Jokela, 2019). The one major advantage of using Bruvo distance is that it can be used to calculate genetic distances regardless of ploidy level (Dufresne *et al.,* 2014). However in mixed ploidy systems (such as in *Rhodohypoxis baurii*), the method assumes autopolyploidization and assigns one or more 'virtual alleles' to the individuals with the lower ploidy level. This would result in incorrect inferences for organism where polyploid individuals are allopolyploids or in systems where hybridization occurs often (Dufresne *et al.,* 2014). The alternative statistic to use instead of Bruvo distance would be *Rho-ST*, which has been favoured as it is the only measure of population differentiation that is independent of the ploidy level or selfing rate (Meirmans & Van Tienderen, 2013). However, *Rho-ST* is analogous to the 'correlation between truly outcrossed mates' defined for diploids (Tachida & Yoshimaru, 1996) and therefore would result in incorrect estimations of population differentiation in asexually reproducing polyploids such as *R. baurii.* Therefore, given the complexity of our mixed ploidy system, Bruvo distance was used to infer genetic differentiation amongst *R. baurii* populations.

Gene flow is another mechanisms that can, and often does, contribute to polyploid genetic diversity (Glennon & Church, 2015; Slotte et al., 2008; Stift et al., 2010). Gene flow between polyploid *R. baurii* populations and between polyploid and diploid individuals within populations of *R. baurii* is considerably high. The Southern Drakensberg and away from the main Drakensberg populations show high amounts of shared alleles between all varieties and ploidy levels. Therefore, gene flow between polyploids as well as between polyploid and diploid lineages in *R. baurii* may be resulting in the high genetic diversity observed here. Interspecific gene flow tends to occur more often between species with the same ploidy level

as observed in *Castilleja* Mutis ex L.f. (Orobanchaceae) (Hersch-Green, 2012) and *Houstonia* L. (Rubiaceae) (Glennon & Church, 2015), and can result in higher genetic diversity in polyploid lineages. Gene flow among polyploid lineages and their diploid progenitors contributing to polyploid genetic diversity has also been seen in other genera such as *Capsella* Medik. (Brassicaceae) where gene flow from diploid species resulted in increased genetic diversity of tetraploid species (Slotte et al., 2008). Therefore, gene flow between various ploidy levels in *R. baurii* individuals and populations may be maintaining high polyploid genetic diversity despite the prevalence of asexual reproduction.

The genetic and reproductive data suggest that interploidy gene flow is occurring between *R. baurii* diploid and tetraploid individuals as the different cytotypes across all *R. baurii* varieties are neither reproductively nor geographically isolated and are sharing alleles. However, gene flow is highest between geographically close populations irrespective of ploidy or taxonomy with geographically proximate populations sharing more alleles. Golden Gate and Sentinel in the Northern Drakensberg have similar allelic diversity, likewise all populations in the Central and Southern Drakensberg have similar allelic diversity and the three Eastern Cape Drakensberg populations have similar allelic diversity. Platberg and Karkloof populations presented as outliers with unique genotypes. Karkloof is a geographically isolated population occurring off the main Drakensberg towards the KZN Midlands and has a different climate and different ecological conditions than the main Drakensberg regions The unique climate and ecology of the Karkloof population is likely the reason that this tetraploid- dominated population of *R. baurii* var. *platypetala* has a unique allelic diversity/profile. Platberg is also a relatively geographically isolated population away from Drakensberg Mountain Centre with two *Rhodohypoxis* species (*Rhodohypoxis baurii* and *Rhodohypoxis milloides* co-occurring in the population. Hybrid individuals have been identified in the Platberg population (Glennon pers. obs) with the three tetraploid *R. baurii* individuals showing intermediate morphology and the alleles present in the population structure analyses shows admixture. Therefore, the mixed unique allelic diversity at Platberg is likely due to hybridisation and introgression occurring between different species and ploidy-levels, resulting in a diverse array of allo-polyploid individuals. However, understanding polyploid genetics using microsatellite markers comes with potential biases in analysing and interpreting the results due to inferring dosage. Reliably resolving the dosage of alleles in polyploid

is needed in order to implement analyses that rely on allele frequency-based inferences (such as Nei's gene diversity) (Dufresne *et al.,* 2014). Furthermore, homoplasy could also result if there are stutter bands caused by replication slippage during the PCR process, which could change the allele frequency distribution and therefore, the inferences about population genetic structure (Dufresne *et al.,* 2014). We have attempted to mitigate these potential biases by scoring the microsatellite peaks manually, and only scoring peaks that are more than 4 base units apart or greater in order to avoid double scoring peaks (scoring null alleles) that had stutters due to replication slippage. Secondly, we used Genodive version 3.05 (Meirmans, 2020) to correct for unknown dosage of alleles rather than rely on dosage interference from peak fluorescent intensity which is notoriously unreliable.

Latitudinal polyploid gradient

Rhodohypoxis baurii populations show a distinct latitudinal ploidy gradient with tetraploid *R. baurii* individuals occurring at higher latitudes (more southerly) in the Drakensberg (ca. 30° S) and populations consisting exclusively of diploid individuals occurring at lower latitudes (ca. 28° S), i.e. more northerly regions. Mixed ploidy populations are present in between these two geographic ploidy zones (at ca. 29° S). Polyploid trends corresponding to latitude have been noted repeatedly in angiosperms (Bottini et al., 2000; Leitch & Bennett, 2004; Leong-Škorničková et al., 2007; Ohri & Khoshoo, 1986; Rice et al., 2019) particularly in the Northern hemisphere. Polyploids have greater genetic and biochemical diversity (Levin, 2002; Otto & Whitton, 2000), allowing for greater ecological tolerance and better colonizing capabilities especially in deglaciated areas (Brochmann et al., 2004; Stebbins, 1984, 1985). Harsher climatic conditions at higher latitudes, such as colder temperatures, might favour polyploids as they are able to better tolerate these stressful environments. Furthermore, the association of polyploids with higher latitudes suggests an increase in cold tolerance or an increased rate of polyploidization due to the cold climatic conditions (Ehrendorfer, 1980; Thompson et al., 2015). This is consistent with the trends observed in *R. baurii* as the higher latitude Eastern Cape Drakensberg populations consist entirely of tetraploid individuals (such as those at Vrederus Trout Farm, Naudes Nek Pass and Tenahead Lodge). They occur in deglaciated areas (Lewis & Illgner, 2001) at higher altitudes (ca. 2300 m a.s.l.) where significantly higher amounts of snow fall over a longer period in the Drakensberg (Mulder & Grab, 2009). The Eastern Cape Drakensberg region experienced late quaternary paleoglaciation with

extensive snowblow (Lewis & Illgner, 2001), which may have promoted *R. baurii* polyploid formation or colonization post-glaciation. Glacial periods in the Drakensberg's history is not restricted to the Eastern Cape Drakensberg as glaciation has been noted in the Southern Drakensberg as well (Mills & Grab, 2005), however the cirque glaciation that occurred in the Southern Drakensberg during the last glacial maximum (LGM) was climatologically restricted to a few sites as conditions were only conducive to small glacial pockets occurring over 3000 m a.s.l.(Mills & Grab, 2005). Therefore, it is unlikely that the Southern Drakensberg populations sampled in this study occur in deglaciated areas, whereas it is likely that the Eastern Cape Drakensberg populations are occurring in a deglaciated area (Lewis & Illgner, 2001; Mills & Grab, 2005), possibly explaining the presences of exclusively tetraploid populations. However, without a dated phylogeny of *Rhodohypoxis,* we cannot correlate the origin of the polyploid lineages to glaciation periods and can only speculate about polyploid formation corresponding to glaciation history of these regions.

Other latitude-related environmental factors, such as precipitation, have also been found to limit the distribution of certain plant groups along a latitudinal gradient. In *Cynoon dactylon* Pers., it was found that precipitation and temperature acting on the growth and development of individuals were the most influential factors affecting the distribution of polyploid populations along a latitudinal gradient (Rice et al., 2019). However, the effects of environmental and climatic variables potentially affecting polyploid distribution in *R. baurii* warrants further investigation.

Latitudinal position and geographic proximity of populations to one another are important characters shaping population genetic structure and polyploidy in *Rhodohypoxis baurii* varieties. The maintenance of genetic diversity is dependent on geographic proximity and isolation of populations (Fehlberg & Ferguson, 2012). The distribution of polyploids has been linked to several landscape attributes with the frequency of polyploids increasing with latitude (Grant, 1981; Love & Love, 1949; Weiss-Schneeweiss et al., 2013), altitude (Husband et al., 2013; Löve & Löve, 1967) and other environmental extremes such as temperature and precipitation (Bottini et al., 2000; Maherali et al., 2009; Stevens et al., 2020). Polyploidy variation together with geographic isolation and distribution may play a role in shaping diversity, genetic differentiation and potentially speciation events in mixed ploidy taxa (Fehlberg & Ferguson, 2012). Ecogeographic divergence allows for niche differentiation which is an important mechanism for polyploid individuals to overcome MCE (Fowler &

Levin, 1984; Levin, 1975; Rodriguez, 1996) and for maintaining genetic stability in populations (Glennon *et al*., 2012).

The latitudinal ploidy gradient seen in *R. baurii* is one of the few documented instances of such a gradient in the Southern hemisphere. Other associations between geographic ranges and ploidy have been noted in southern Africa before, such as in the polyploid *Schoenus* (Cyperaceae) species occurring in drier locations with more variable precipitation (Elliott et al., 2023) and individuals with smaller chromosomes having wider geographic ranges and niche sizes (Elliott et al., 2022). The ecogeographic separation of polyploid populations from diploid populations resulting in the observed latitudinal gradient is likely an important mechanism that enabled polyploid survival and establishment in *R. baurii,* and may result in lineage divergence in *R. baurii* with the Eastern Cape tetraploid *R. baurii* var. *baurii* diverging from all other populations and varieties. However, more extensive sampling (increase number of populations and individuals) is needed to confirm this pattern and to evaluate if it is consistent across the entirety of *R. baurii'*s range.

Taxonomic implications

Rhodohypoxis baurii varieties were evaluated using morphological, molecular and geographic tools to better recognise and distinguish the three varieties. Overall, the varieties do not differ greatly, except for the "on the main berg" Eastern Cape Drakensberg *R. baurii* var. *baurii* which is addressed in subsequent paragraphs.

The morphological NMDS showed overlap between all three *R. baurii* varieties, with *R. baurii* var. *confecta* and *R. baurii* var. *baurii* varieties overlapping greatly and *R. baurii* var. *confecta* and *R. baurii* var. *platypetala* also overlapping. The NMDS showed little overlap between *R. baurii* var. *baurii* and *R. baurii* var. *platypetala. Rhodohypoxis baurii* var. *platypetala* showed the widest morphological variation, likely due to the multiple ploidy levels within the variety. It is likely that the morphological overlap seen between *R. baurii* var. *confecta* and *R. baurii* var. *platypetala* is due to ploidy levels, with like-ploidy individuals of *R. baurii* var. *confecta* and *R. baurii* var. *platypetala* sharing similar morphology, and polyploid *R. baurii* var. *platypetala* being morphologically distinct from both diploid *R. baurii* var. *confecta* and diploid *R. baurii* var. *platypetala.* However, this

cannot be confirmed as flow cytometry could not be conducted on herbarium specimens due to the age of the tissue.

Certain traits differed between all three of the varieties namely flower colour, peduncle length and flower size, yet these differences can be linked to differences in ploidy-levels or are likely due to differences in altitude and/or environmental factors. In general, *R. baurii* var. *confecta* and *R. baurii* var. *baurii* are smaller in form, with smaller tepals and shorter peduncles than *R.* var. *platypetala.* The main distinguishing features between the three varieties are flower size, and flower colour. *Rhodohypoxis baurii* var. *platypetala* has significantly larger flowers (larger tepal length and width) than both *R. baurii* var. *confecta* and *R. baurii* var. *baurii.* However, polyploid *R. baurii* individuals in the Southern Drakensberg populations have larger flowers than diploid individuals, and *R. baurii* var. *platypetala* contains the highest number of polyploid individuals, therefore, the differences in flower size between *R. baurii* var. *platypetala* and the other two varieties is likely due to differences in ploidy. As noted above, the tetraploid *R. baurii* var. *baurii* in the Eastern Cape Drakensberg are morphologically distinct from all other varieties and populations and do not exhibit larger floral or reproductive traits like the other *R. baurii* polyploids found in the Northern, Central and Southern Drakensberg. The tetraploid *R. baurii* var. *baurii* occurring in the Eastern Cape Drakensberg have smaller flowers (shorter tepals), thinner leaves, shorter peduncles and have smaller anthers. *Rhodohypoxis baurii* var. *platypetala* individuals have longer peduncles than both *R. baurii* var. *confecta* and *R. baurii* var. *baurii* occurring away from the Drakensberg. However, the differences in peduncle length are likely a result of altitude as peduncle lengths of all *R. baurii* varieties decrease with an increase in altitude and *R. baurii* var. *platypetala* occurs at lower altitudes than *R. baurii* var. *confecta* and *R. baurii* var. *baurii*. *Rhodohypoxis baurii* var. *platypetala* has shorter wider leaves than *R. baurii* var. *baurii* occurring away from the Drakensberg and *R. baurii* var. *confecta.*

The DAPC grouped by taxonomy indicates that the three *R. baurii* varieties are somewhat genetically distinct with *R. baurii* var. *confecta* and *R. baurii* var. *platypetala* overlapping slightly. The STRUCTURE analysis shows that genetic differences between the three varieties are linked to geography, with geographically proximate populations showing similar genotypes irrespective of the variety or ploidy-levels present in said populations. The Northern Drakensberg populations (Sentinel and Golden Gate) consisting of *R. baurii* var. *confecta* and *R. baurii* var. *platypetala* show nearly identical genotypes with many shared

alleles present and high levels of gene flow resulting in very similar genetic profiles, indicating that the varieties present at these populations are the same taxon, and differences in morphology seen are likely due to altitude. The population of *R. baurii* at Golden Gate as originally identified as *R. baurii* var. *platypetala* by Hilliard and Burtt (1978) and did exhibit the usually *R. baurii* var. *platypetala* morphology and habitat, however our molecular and morphological analyses suggest that's the variety at Golden Gate is more likely to be *R. baurii* var. *confecta.* Further, the Golden Gate population had been identified as *R. baurii* var. *platypetala* mainly due to the white flower phenotype that the population exhibited consistently each year. However, in 2021-2022 flowering season, the Northern Drakensberg (Golden Gate and Sentinel) received higher than usual rainfall, resulting in the usually white flowered Golden Gate population consisting of predominantly pink flowers. An important distinguishing feature between *R. baurii* var. *platypetala* and *R. baurii* var. *confecta* is flower colour, however Gardiner & Glennon (2019) showed that flower colour in *R. baurii* var. *confecta* is likely a function of soil moisture and an additional study showed that the flower colour of *R. baurii* var. *confecta* is linked to soil moisture and UV exposure with wetter soil and higher UV exposure resulting in increased frequency of pink morphs (Mtileni et al., 2022, in review). Therefore, it is highly likely that the variety present at Golden Gate is *R. baurii* var. *confecta* and not *R. baurii* var. *platypetala*. The analyses conducted in this study classified the 7 Golden Gate individuals present in our data set as *R. baurii* var. *platypetala* which is likely an incorrect classification based on the molecular results.

Therefore, these ecological influences (such as rainfall and UV) have resulted in the recognition of these two varieties, *R. baurii* var. *platypetala* and *R. baurii* var. *confecta*, which are somewhat morphologically and genetically distinct, and usually occupy distinct ecological niches, although apparently not at Golden Gate. Additionally, *R. baurii* var. *platypetala* and *R. baurii* var. *confecta* varieties co-occur at Langalibalele Pass (Giants Castle, Central Drakensberg) where *R. baurii* var. *platypetala* occurs at lower altitudes (1000–2000 m a.s.l.) and transitions into *R. baurii* var. *confecta* occur at higher altitudes (2100–3000 m a.s.l.) (Glennon pers. obs). The Karkloof *R. baurii* var. *platypetala* population exhibits a unique genotype and the Platberg population shows an admixed genotype as discussed previously.

The Central and Southern Drakensberg and 'away from the main Drakensberg' populations that comprise all three varieties are not genetically district, indicating that allele sharing and

gene flow is occurring between all varieties across these populations. The geographically isolated Eastern Cape *R. baurii* var. *baurii* are genetically distinct from all other populations and varieties indicating a lack of gene flow between these more northerly populations and those at/near the southern tip of the Drakensberg. Geographically, the three varieties are not isolated with *R. baurii* var. *platypetala* and *R. baurii* var. *confecta* populations sometimes occurring in the same regions.

Hilliard and Burtt (1987) noted two distinct morphological forms in *Rhodohypoxis baurii* var. *baurii* associated with geographic distribution. The first form occurs "away from the main Drakensberg", at lower altitudes (1140 – 1740 m a.s.l.) and has wider leaves, whilst the other form occurs "on the main Drakensberg" or "facing the main Berg at heights" and occurs at higher altitudes (2070 – 2640 m a.s.l.) and has thinner leaves. Our data corroborate Hilliard and Burtt's (1987) observations, as the *R. baurii* var. *baurii* populations (e.g., Hebron Farm) away from the main Drakensberg have wider, longer leaves and have longer and wider tepals compared to the *R. baurii* var. *baurii* populations on the main berg in the Eastern Cape Drakensberg (e.g., Vrederus, Naude's Nek, Tenahead). Not only are the "main Drakensberg" *R. baurii* var. *baurii* that occur in the Eastern Cape Drakensberg morphologically and geographically distinct, but they are also genetically distinct from all other *R. baurii* varieties. Furthermore, seed set was lowest in interspecific crosses when *R. baurii* var. *baurii* from the main Eastern Cape Drakensberg population was the maternal plant, indicating a degree of reproductive isolation between the Eastern Cape *R. baurii* var. *baurii* and all other *R. baurii* populations. The morphological and molecular data support Hilliard and Burtt's (1987) suggestion regarding two distinct *R. baurii* var. *baurii* morphs occurring on the main Drakensberg and away from the main Drakensberg. Additional work is needed to better delineate these morphs within *R. baurii* var. *baurii.*

During glacial periods, the distribution of the high altitude *R. baurii* var. *baurii* in the Eastern Cape may have migrated downwards into lower altitudinal areas that tend to be warmer and more hospitable. This could have formed the "away from the Berg" population which is now genetically and morphologically distinct from the original population. Remnants of the high altitude population of *R. baurii* var. *baurii* may have survived in protected pockets due to adaptive advantages brought upon by a prior polyploidisation event. These remnants over time may have expanded into the original range post-glaciation and have since become more genetically distinct. Therefore, polyploid advantage may have occurred in *R. baurii* var.

baurii, with shifts in climate, due to the glacial periods, , resulting in only the minority cytotype tetraploids surviving in this Eastern Cape Drakensberg population, persisting and subsequently establishing and outcompeting the diploid cytotype.

Sympatric occurrence of diploids and tetraploids at the same site results in MCE for the minority cytotype (Castro et al., 2019; Kolář et al., 2017; Levin, 1975). Recurrent polyploid formation in diploid populations results in MCE for tetraploids and would hamper common and long-term cytotype co-existence unless a different habitat or niche is reached by the minority polyploid cytotype (Castro et al., 2019; Kolář et al., 2017; Levin, 1975). Therefore, niche differentiation and/or geographic isolation likely occurred between tetraploid *R. baurii* var. *baurii* populations found in the Eastern Cape Drakensberg and the other *R. baurii* populations. Higher ploidy levels are more adaptable to different or new conditions due to genetic advantages that facilitate establishment and persistence (Comai, 2005; Liu & Sun, 2019; Stevens et al., 2020; Van de Peer et al., 2021; Wei et al., 2019). Polyploids that are better adapted to a broader degree of ecological conditions will have a competitive advantage resulting in the ability to move into new niches or withstand harsh or new climatic conditions (Maceira et al., 1993; Petit et al., 1999; Soltis & Soltis, 2000; Van de Peer et al., 2021; Wei et al., 2019).

Therefore, ecogeographic isolation may have acted as an important reproductive barrier between polyploids and diploids in *R. baurii*, playing a role in lineage divergence and polyploid speciation events. After the initial reproductive isolation (indicated by the lower seed set and germination rates of inter-ploidy crosses) of polyploid *R. baurii* from diploid *R. baurii* (assuming *R. baurii*'s progenitors were diploid), genetic differentiation may have been maintained by spatially restricted gene flow as geographically neighbouring lineages are genetically more similar than spatially separated ones. Gene flow within same (now isolated) ploidy levels has likely lead to the generation of a novel variety in *R. baurii.*

CHAPTER 5: CONCLUSION

Polyploidy has clearly influenced reproductive strategies, morphology and genetic patterns in *R. baurii.* Several morphological differences are present between diploid and polyploid individuals across *R. baurii* but the morphological effects of polyploidy seem to be geographically linked. Polyploid individuals occurring in the Central and Southern Drakensberg and "away from the main Berg" populations tend to have larger tepals, anthers and longer peduncles than diploid *R. baurii* individuals (i.e. exhibiting the Gigas effect). However, polyploid (tetraploid) *R. baurii* var. *baurii* populations located in the Eastern Cape Drakensberg tend to have smaller morphological features than most *R. baurii* diploids, with Eastern Cape Drakensberg polyploid populations having thinner leaves, smaller flowers and shorter peduncles.

A latitudinal polyploidy gradient is apparent with diploid-only populations occurring in the Northern Drakensberg, mixed ploidy populations occurring in the Central, Southern and away from the main Drakensberg population, and exclusive tetraploid populations found in the Eastern Cape Drakensberg. Like-ploidy populations that are geographically close show high levels of gene flow, as seen by their similar genotypes. However, polyploid and diploid *R. baurii* individuals across all varieties are not genetically distinct in the Southern Drakensberg with gene flow occurring between multiple populations, between all ploidylevels and varieties. *Rhodohypoxis baurii* polyploids and diploids are therefore not reproductively isolated as inter-ploidy crosses yielded many viable seeds. However, triploid individuals showed low pollen viability and yielded fewer viable seeds. These *R. baurii* triploid individuals are however/nonetheless not completely reproductively isolated and may be acting as triploid bridges between diploid and tetraploid individuals. High success of inter-varietal and inter-ploidy crosses for certain populations indicate an increase in assortative mating, which may result in polyploid survival and establishment by evading minority cytotype exclusion (MCE). However, polyploid *R. baurii* individuals are also showing a shift away from sexual reproduction to asexual reproduction, with higher instances of clonality noted in polyploid individuals and mixed ploidy populations. Therefore, a shift to clonal reproduction **or** an increase in assortative mating adopted by different polyploid populations of *R. baurii* may be mechanisms by which *R, baurii* polyploids are escaping MCE.

Taxonomically, the tetraploid populations of *R. baurii* var. *baurii* located in the Eastern Cape Drakensberg "on" and "off" the main escarpment seem to be ecologically, geographically, genetically and morphologically distinct, and further investigations are needed to identify if these tetraploid populations of *R. baurii* var. *baurii* are a new or novel lineage. *Rhodohypoxis baurii* var. *confecta* and *R. baurii* var. *platypetala* are genetically, geographically and morphologically similar, differing only in flower colour, flower size and peduncle length. However, most of these differences can be attributed to differences in ploidy and /or altitude with *R. baurii* var. *confecta* occurring at higher altitudes and *R. baurii* var. *platypetala* containing multiple ploidy-levels. It is therefore evident that ecological differences have shaped these two taxa and changes in ploidy have also played a role.

Geographic location and isolation have also played a large role in the genetic structure of *R. baurii* populations and varieties, with geographically isolated populations such Karkloof and Platberg exhibiting unique genotypes and phenotypes. The geographic isolation and subsequent reproductive isolation of polyploid populations (such as the Karkloof and the three Eastern Cape populations) resulting in unique genotypes has likely lead to lineage divergence in these populations. On the contrary, high inter-varietal and inter-ploidy gene flow and hybridisation among (and within) other populations such as Platberg and those occurring in the Central and Southern Drakensberg may be leading to lineage homogenization. Finally, there is evidence that shifts in reproduction and morphology due to polyploidy are resulting in changes in gene flow that could promote both lineage divergence and homogenization in *R. baurii* varieties.

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APPENDIX

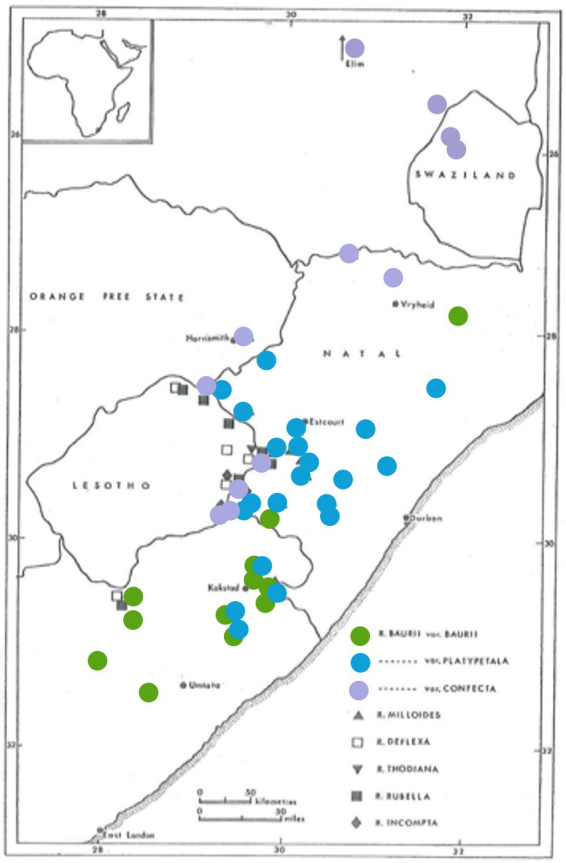


Figure 1. Distribution map of *Rhodohypoxis* species (Hilliard & Burtt, 1978) with *Rhodohypoxis baurii* varieties highlighted in colour (var. *baurii* = green, var. *platypetala* = blue and var. *confecta* = *purple).*

Table 1: Herbarium specimens of *R. baurii* varieties included in the morphometric data analyses. Identification code used, collector, collection date and geographical locality indicated.

Code	Specimen collection	Coll. date	Locality
Hbp1	*Hilliard, O.M. & Burtt, B.L. 7550* (NU)	1973	Natal-Transkei border, Alfred district, Zuurberg
Hb2	*Grice, M. (*NU)	1973	Underberg district, Bamboo Mountain
Hb3	*Hilliard, O.M. & Burtt, B.L.* 7318A (NU)	1973	Transkei, Mt. Auliff district
Hb4	*Strey, R.G. 9177* (NU)	1969	Transkei, 3029BC, Kokstad, Sneezewood Plantation
Hb5	*Van Wyk., P 7469* (NU)	1986	KZN, Weza State forest, Ngeli Mountains, Foothills
Hb6	*Sikhakhane, T.B. 1230* (PRE)	1993	Mzimkhulu district, Nsikeni Nature Reserve,
Hb8	*Hilliard, O.M. & Burtt, B.L. 7549* (NU)	1973	Alfred district
Hp9	*Hilliard, O.M. & Burtt, B.L. 3469* (NU)	1966	Alfred district, Ngeli Mt.
Hbp10	*Hilliard, O.M. & Burtt, B.L. 2532* (NU)	1964	Natal, Alfred district, Mpetyne Forest
Hpm11	*Hilliard, O.M. & Burtt, B.L. 7314* (NU)	1973	Transkei Mt. Auliff distr. Mt Insiziwa
Hpm12	*Hilliard, O.M. & Burtt, B.L. 7354* (NU)	1973	Transkei, Tabankulu Mt., cliffs east of road to Port St. John
Hpm13	*Hilliard, O.M. & Burtt, B.L. 7335* (NU)	1973	Transkei, Tabankulu Mt.
Hb14	*Taylor, B. 254* (NU)	1991	Mt. Currie 30º30'S,24º29'E

Hpm15	*Hilliard, O.M. & Burtt, B.L. 8399* (NU)	1975	Natal, Nqothe distr., Mt. Ngome

Hb16	*Hilliard, O.M. & Burtt, B.L. 18991* (NU)	1986	Natal, 2929cc, Manguinubu river, below farm Hinton
Hpm17	*Hilliard, O.M. & Burtt, B.L. 9551* (NU)	1976	Natal, Underberg distr., Coleford Nature Reserve
Hb18	*Stewart 1837* (NU)	1976	Underberg district, the farm "Lemmermoor", Banks of Ngwangwana River
Hpm19	*Grice, M. (*NU)	1973	Underberg district, Bamboo Mt.
Hc21	*Hilliard, O.M. & Burtt, B.L. 14548* (NU)	1981	Transkei, 3127DB, Satanna's Nek
Hb22	*Koekemoer 1637* (PRE)	1999	Between Rhodes and Naudes Nek Pass
Hc23	*Davis, L. & Davis, R. 180* (NU)	1979	Lesotho, Sehlabethebe National Park
Hb24	*Acocks, 12183* (PRE)	1945	Naudes Nek Pass, Maclear district
Hpm29	*Hilliard, O.M. & Burtt, B.L. 6964* (NU)	1973	Estcourt district, "Game Pass" & Highmoor Forest Reserve.
Hmp30	*Hilliard, O.M. & Burtt, B.L. 9094* (NU)	1976	Natal, Lions river district, Nhlugane
Hp31	*Hilliard, O.M. & Burtt, B.L. 9093* (NU)	1976	Natal, Lions river district, Nhlugane
Hp32	*Potgieter, 843* (NU)	2002	Minerva Private Nature Reserve, near Richmond, KZN.
Hc33	*Devenish, 1561* (NU)	1975	Oshoek, Wakkerstroom district.
Hp34	*Schlechter, 2279* (PRE)	1895	Zuurbergen
Hp35	*Arnold, 540* (PRE)	1973	Natal, 20km from Himeville on road to Sani Pass.
Hp36	*Symons, 187* (PRE)	NA	2929AD, Giants Castle
Hp37	*Werdermann & Oberdieck, 1368* (PRE)	1958	Natal, Underberg distr., Drakensberg, Himeville.
Hp38	*Edwards, 2169* (PRE)	1957	Giants Castle Reserve, Estcourt distr. 2929AB.
Hppv39	*Hilliard, O.M. & Burtt, B.L. 7311* (PRE)	1973	Mt. Auliff distr. Transkei
Hp40	*Potgeiter, 753* (PRE)	2002	Blinkwater Trails, area near Greystown, KZN
Hp41	*Stalmans, M. 2880* (J)	1993	Transvaal, Barberton District, Songimvelo Game Reserve, on farm Schoonoord, 2531CC
Hp42	*Johnson & Neal, 24* (NU)	2000	Summit of Mt. Gilboa, Karkloof Mountain range, KZN
Hp43	*Johnson & Neal, 24* (NU)	2000	Summit of Mt. Gilboa, Karkloof Mountain range, KZN
Hb44	*Brand, Swart & Smith, 442* (NU)	2001	KZN, Ngeli Forest. 100–200m west of lookout. 30º31.273'S, 29º41.448'E
Hp45	*Wright, 2226* (NU)	1975	Lions River distr., Kamberg, South facing slope
Hc47	*Schmitz, 8833* (NU)	1979	Lesotho, 10km before Ramatselisos gate.
Hp48	*Edwards, 2169* (NU)	1957	Giants Castle Reserve, Estcourt distr.
Hp49	*Hilliard, O.M. & Burtt, B.L. 7365,* (NU)	1973	Farm Hebron. NE of Kokstad
Hp50	*Hilliard, O.M. & Burtt, B.L. 7374* (NU)	1973	Farm Hebron. NE of Kokstad
Hp51	*Hilliard, O.M. & Burtt, B.L. 7364* (NU)	1973	Farm Hebron. NE of Kokstad
Hp53	*Hilliard, O.M. & Burtt, B.L. 7547* (NU)	1973	Natal-Transkei border, Alfred district, Zuurberg
Hp54	*Hilliard, O.M. & Burtt, B.L., 7548* (NU)	1973	Natal-Transkei border, Alfred district, Zuurberg
Hp55	*Hilliard, O.M., 2065* (NU)	1964	New Hammer distr., Noodsburg, Kingscliffe.
Hb56	*Grice,* (NU)	1973	Underberg distr, Bamboo Mountain
Hpm57	*Grice,* (NU)	1973	Underberg distr, Bamboo Mountain
Hp58	*Hilliard, O.M. & Burtt, B.L., (7399)* (NU)	1973	Underberg distr, Bushmans Nek
Hpm59	*Hilliard, O.M. & Burtt, B.L., 7213* (NU)	1973	Sunset Farm, Polela distr.
Hpm61	*30* (NU)		Natal, Underberg distr., Sani Pass
Hpm62	*Hilliard, O.M. & Burtt, B.L,. 7022* (NU)	1973	Underberg distr., Drakensberg Gardens, Forest Reserve.
Hp63	*Goodies, 372* (NU)	1957	Cathedral Peak area.
Hpb64	*Sabal, 827* (NU)	1944	Bamboo slopes, Cathedral area.
Hpm65	*Hilliard, O.M. & Burtt, B.L,. 6916* (NU)	1973	Bergville distr., Cathedral Peak Forestry Reserve.
Hp66	*Hilliard, O.M. & Burtt, B.L,. 6968* (NU)	1973	Estcourt distr., "Game Pass" and Highmoor Forest Reserve.
Hp67	*Gardau-Gray, 108* (NU)	1947	Natal, "Game Pass"
Hp68	*Hilliard, O.M. & Burtt, B.L., 6963* (NU)	1973	Estcourt distr., "Game Pass" and Highmoor Forest Reserve.
Hpm69	*Morris, 146* (NU)	1963	Impendle distr., Elandskop.

Hp70	*Hilliard, O.M. & Burtt, B.L., 9099* (NU)	1976	Lions River distr., Nhlugane
Hpm71	*Grove, 43* (NU)	1987	Natal: 25KM NNE of Howick, Twin Falls. 29º16’20”S, 30º19’40”E.
Hpm72	*Hilliard, O.M. & Burtt, B.L., 11848* (NU)	1978	Natal, 2930AD, Lions River distr. Mt Gilboa.
Hp73	*Hilliard, O.M. & Burtt, B.L., 9098* (NU)	1976	Lions River Distr., Nhlugane

Hp74	*Moll, 1131* (NU)	1964	Zwartkop Location. Pietermaritzburg, Lions river district.
Hppv75	*Hilliard, O.M. & Burtt, B.L., 7352*	1973	Transkei, Tabankulu Mountain.
Hp76	*Hilliard, O.M., 5585* (NU)	1975	Richmond distr., Escarpment above Byrne valley
Hp77	*Stewart, 1693* (NU)	1973	Richmond: Hilltops above Byrne
Hpm78	*Stewart, 1695* (NU)	1973	Richmond: Hilltops above Byrne
Hp79	*Hilliard, O.M. & Burtt, B.L., 9127* (NU)	1976	Richmond distr., Peak of Byrne
Hp80	*Hilliard, O.M. & Burtt, B.L., 7316* (NU)	1973	Mt. Auliff distr. Mt. insizwa. Transkei
Hp81	*Hilliard, O.M. & Burtt, B.L., 7313* (NU)	1973	Mt. Auliff distr. Mt. insizwa. Transkei
Hpm82	*Hilliard, O.M. & Burtt, B.L., 7311* (NU)	1973	Mt. Auliff distr. Mt. insizwa. Transkei
Hp83	*Cowan, 146* (NU)	1978	Natal, Underberg dist., Cobham Farm, Palela Valley
Hp84	*Huntley, 448* (NU)	1948	Natal, Impendle
Hp85	*Wright, A1* (NU)	1976	Lions River distr., Fort Nottingham
Hp86	*Hilliard, O.M. & Burtt, B.L., 9064* (NU)	1976	Lions River distr., Fort Nottingham
Hp87	*Frankish, M.S. 187* (NU)	1957	Natal, Giants Castle Game Reserve
Hp88	*Wright, 1605* (NU)	1973	Estcourt Distr., Giants Castle Game Reserve
Hpm89	*Wright, 1289* (NU)	1972	Estcourt Distr.
Hp90	*Skead, 198* (NU)	1961	Estcourt Distr., Giants Castle Game Reserve
Hp91	*Wright, 1903* (NU)	1974	Lions River distr., Kamberg
Hp92	*Smook, K. 519* (NU)	1973	Lions river distr., Dougle farm
Hp93	*Wright, 1562* (NU)	1973	Lions River distr., Kamberg
Hpm94	*Hilliard, O.M. & Burtt, B.L., 6981* (NU)	1972	Lions river distr., Dougle farm
Hcp95	*Hilliard, O.M. & Burtt, B.L., 7353* (NU)	1973	Transkei, Tabankulu mt.
Hpm96	*Balkwill, K. 5195* (J)	1990	Natal, Mooi River District, Farm Game Pass. 2929BC
Hppv97	*Boardman, 125* (PRE)	1976	Transkei, 3028BB
Hc99	*Hilliard, O.M. & Burtt, B.L., 8685* (PRE)	1975	Royal Natal National Park
Hb100	*Comté, R.H. 26108* (PRE)	1956	Abneis Farm, Mbabane
Hp101	*Singh, Y. 458* (PRE)	1998	28º41"31'S, 28º53"36'E (2828DB). Qwaqwa. Qwaqwa National Park- Sentinel Peak.
Hpm102	*Browning & Paton, 13* (NU)	1986	Fiko Palso, Qwa-Qwa. E28º50'S, 28º40'
Hp103	*Balkwill, K. & Balkwill, M.J. 5193* (J)	1990	KZN, Mooi river district, Farm Game Pass.
Hcm104	*Hilliard, O.M. 517* (NU)	NA	Natal, Bergville distr., Cathedral Peak area
Hc105	*Hilliard, O.M. 5414* (NU)	1973	Lesotho, Sani Top
Hc106	*Browning & Paton, 4* (NU)	1986	Fiko Patso, Qwaqwa. E28º50', S28º40'
Hc107	*Boardmans, 170* (NU)	1976	Lesotho, Ramatsiliso's Beacon
Hc108	*Schmitz, 8809* (NU)	1979	Lesotho, Sehlabethebe
Hbpv109	*Boardmans, 168* (NU)	1976	Transkei, Matatiele distr.,
Hc110	*Hilliard, O.M. & Burtt, B.L., 7462* (NU)	1973	Underberg distr., Bushmans Nek
Hcd111	*Hilliard, O.M. 5332* (NU)	1973	Lesotho, Sani Pass
Hcpv112	*Boardmans, 169* (NU)	1976	Lesotho, Ramatsiliso's Beacon
Hc113	*Balkwill, Manning & Meyer, 1077A-D* (J)	1983	Natal Drakesnberg, spur SE of Cathkin Peak
Hc114	*Hilliard, O.M. & Burtt, B.L., 9615* (NU)	1977	Underberg distr., Sani Pass
Hcp115	*Browning & Paton, 13* (NU)	1986	E28º50', S28º40'. Fiko patso QwaQwa. South Africa
Hc116	*Hilliard, O.M. & Burtt, B.L., 9170* (NU)	1976	Utrecht distr., Farm "Naauwhoek"
Hc117	*Hilliard, O.M. & Burtt, B.L., 8685* (NU)	1975	Royal Natal National Park
Hpm118	*Wright, 1316* (NU)	1972	Impendle distr., Hlatimbe pass, Lesotho summit plateau

Hc119	*Davis, L. & Davis, R., 196* (NU)	1979	Natal, top of Bushmans Nek Pass
Hcpv121	*Hoener, 1597* (NU)	1976	Sehlabathebe National Park, Mokhoabong
Hcpv122	*Galpin, 10136* (NU)	1928	Mount Aux Sources
Hc123	*Hilliard, O.M. & Burtt, B.L., 7432* (NU)	1973	Underberg distr., Bushmans Nek,
Hpm124	*Hilliard, O.M. & Burtt, B.L., 9189* (NU)	1976	Wakkerstroom Distr.
Hc125	*Hilliard, O.M. & Burtt, B.L., 13772* (NU)	1980	Natal, Underberg distr., 2929CC, Garden Castle Forest Reserve.
Hp126	*Hilliard, O.M. & Burtt, B.L., 8693* (NU)	1975	Harrismith distr., Platberg.

Table 2. Sixty *Rhodohypoxis* microsatellite primers were developed by AllGenetics (AllGenetics & Biology SL, A Coruña, Spain), 28 were successfully amplified and optimized listed below. The 12 most polymorphic markers, ID = 1-12 (bolded), were used in this study.

ID	Locus	Motif	Amplicon size (bp)	Forward primers (5'-3')	Reverse primers (5'-3')	Fluorescent label	Tails	Annealing temperature	# cycles	Amplicon range
1	**AG_Rba_182**	AG	144	AATGGCTGACACCAACTTCC	GGAATGACATCAGCCCAAAC	FAM	CAG	57.2	30-33	95-190
2	**AG_Rba_473**	AG	153	ATCTCGATGGTGATCCAACC	TAATCCAAAGCGGAGACCAT	FAM	CAG	57.2	30-33	140-190
3	**AG_Rba_290**	AG	121	ACAGGAGTGCATCCTTCGTT	GGGTTATGCACATGGGTAGC	FAM	CAG	57.2	30-33	110-170
4	**AG_Rba_489**	CCCGG	123	ATGTGTAGACACGGCACGAC	AATGCTAGTGACAGCGACGA	FAM	CAG	57.2	30-33	115-155
5	**AG_Rba_304**	AC	104	TTGCTGCCGAGACATAATTG	GAGGTCACAGGCTCAATTCC	FAM	CAG	57.2	30-33	97-230
6	**AG_Rba_206**	AG	152	CCTACGGGTCCATAGCTCAG	GTCCGAAGATTCCCAAACAA	FAM	CAG	57.2	30-33	145-190
7	**AG_Rba_307**	AG	129	ACCCATTGTTCCTCCTTCCT	AATGAGTTGCAGTGCACCAA	NED	T3	57.2	30-33	130-190
8	**AG_Rba_165**	AAC	132	CGACGAAATGGTCGACACTA	AGCCCTTCTTCTCCGACTTC	NED	T3	57.2	30-35	120-170
9	**AG_Rba_448**	ATC	91	CCATAGCTGATCCCATTGCT	GGTGTTCCATTGGACGTCAT	NED	T3	57.2	30-35	95-150
10	**AG_Rba_158**	AG	161	ATTAGGGTGCAAATGGATCG	TGTTGGGTGGAAGCTTTAGG	NED	T3	57.2	30-35	160-190
11	**AG_Rba_444**	ATC	155	ATGGAAGTTAGGGTTTCGGC	GCACTCTTCGTTTCAGCCTT	NED	T3	57.2	30-35	150-195
12	**AG_Rba_041**	AAC	143	CTGGCCAAGGCAACATAAAT	GCGCGAGATAGAGCTAATGG	NED	T3	57.2	30-35	140-190
13	AG_Rba_430	AG	122	GGGAGAGGGAGGAAGAAAGA	GGTGACTCCTCTCGAGTGCT	FAM	CAG	57.2	30-33	95-170
14	AG_Rba_009	AAC	103	CGGAGAGCGTATGGCTTTAG	CCTTCTTGCCCATAACCAAA	FAM	CAG	57.2	30-35	95-120
15	AG_Rba_235	AGG	119	GTCTTGGTCATGGTGGGTTC	GTTCTCGAGGAACGGAATCA	FAM	CAG	57.2	30-35	100-160
16	AG_Rba_056	ACTCC	94	ACCCTTCAGCCAAGTGTTTG	AGTAGACGGGTACATTGCCG	FAM	CAG	57.2	30-35	80-170
17	AG_Rba_438	AC	104	TGGAGGAGAAATTGGTGAGC	ATCTGTTGCATGGATTGCCT	FAM	CAG	57.2	30-35	80-195
18	AG_Rba_421	AG	135	TCCTTCTCTTCAGGTCGGAG	GTCCCTCCCTCTTTCTTGCT	FAM	CAG	57.2	30-35	85-170
19	AG_Rba_081	AG	95	CATGCCTTCTTACTCGCCTC	AGAGAAGAAGGGACCTTGCC	FAM	CAG	57.2	30-35	100-220
20	AG_Rba_413	AG	203	AAGGCCGACCTTACCTGAAC	GATTGCCATATGGACTTGGG	FAM	CAG	57.2	30-35	100-230
21	AG_Rba_225	AG	159	GGCTGGACCGAGTGACTTAC	GGAAAGGGATGGATCTTGGT	NED/ HEX	T3	57.2	30-33	120-240
22	AG_Rba_467	AG	106	GAGCTAAACCGTACTCGCCA	AGACAATCCTCCAACCCTCC	NED/ HEX	T3	57.2	30-35	110-200
23	AG_Rba_197	AAG	128	CCACACCTCATCTTCTGGCT	GGTCAGTTCCGGTTTAAGCA	NED/ HEX	T3	57.2	30-35	120-160
24	AG_Rba_460	AG	90	CCATCTTGGAGGCAACAACT	CAAGTTGCATACCAATCCCA	NED/ HEX	T3	57.2	30-35	90-120
25	AG_Rba_225	AG	159	GGCTGGACCGAGTGACTTAC	GGAAAGGGATGGATCTTGGT	NED/ HEX	T3	57.2	30-35	110-240
26	AG_Rba_359	AG	181	AATCCCGAACTTCCAACCTC	TGCGTAATGGTGGAGACAAG	NED/ HEX	T3	57.2	30-35	170-250
27	AG_Rba_004	AG	96	ATAGTGGCACCGGATCTGAC	CCCAGAGGAGATGATCCAAA	NED/ HEX	T3	57.2	30-35	110-190
28	AG_Rba_059	AT	139	GTTGGACCCTGAATCGAGAA	AGTACCTTCCACAACGGGTG	NED/ HEX	T3	57.2	30-35	100-200

Table 3. Principal Component Analysis (PCA) eigenvalues of principal components 1–4 for 23 morphological features across 126 specimens. Traits that exhibited very low eigenvalues (<0.0001), indicating a lack of variability, were removed from the data set (Highlighted/bolded).

Traits	PC 1	PC 2	PC 3	PC 4
Peduncle length (mm)	0.74869	-0.659	-0.062067	-0.022842
Leaf lamina length (mm)	0.66028	0.74924	-0.011993	0.024267
Leaf lamina width (at widest point) (mm)	0.013112	-0.040931	0.18553	0.68157
Peduncle trichome length (mm)	**0.0066671**	**<0.00001**	**0.043491**	**0.11437**
Receptacle trichome length (mm)	0.0057881	0.0035657	0.040069	0.10444
Leaf trichome length (mm)	0.0075332	-0.002385	0.012101	0.1898
Tepal trichome length (mm)	**0.0050614**	**0.0045122**	**0.032141**	**0.075281**
Flowers/ peduncle (mm)	**0.0038391**	**-0.003091**	**-0.003082**	**-0.033979**
Outer tepal length (mm)	0.051397	-0.008381	0.84468	-0.21173
Outer tepal width (mm)	0.017387	-0.01814	0.44116	0.029903
Outer tepal ratio	-0.0012053	0.0062547	-0.011077	0.033258
Trichome density on peduncle	**<0.00001**	**0.0068176**	**0.039185**	**0.067281**
Trichome density on leaf lamina	-0.099742	-0.011728	0.018058	0.0062732
Trichome density on receptacle	**0.00577**	**<0.00001**	**-0.004041**	**0.10379**
Trichome density on tepal	-0.00167	-0.008703	0.041879	0.061329
Trichome branching pattern peduncle	**<0.00001**	**<0.00001**	**0.0075941**	**-0.007759**
Trichome branching pattern leaf lamina	0.070058	0.02128	-0.088277	-0.60473
Trichome branching pattern leaf margin	**<0.00001**	**<0.00001**	**-0.003149**	**-0.001499**
Trichome branching pattern tepal	**<0.00001**	**<0.00001**	**0.0002357**	**-0.010466**
Flower colour	-0.0058226	0.036102	-0.17241	0.14198
Spiky sheaths	**-0.0030263**	**<0.00001**	**0.012875**	**-0.011366**
Tufted trichomes on tepal tips	**<0.00001**	**<0.00001**	**-0.008781**	**0.073651**
Trichome density pattern on leaves	-0.0045097	-0.011669	0.084891	-0.10001

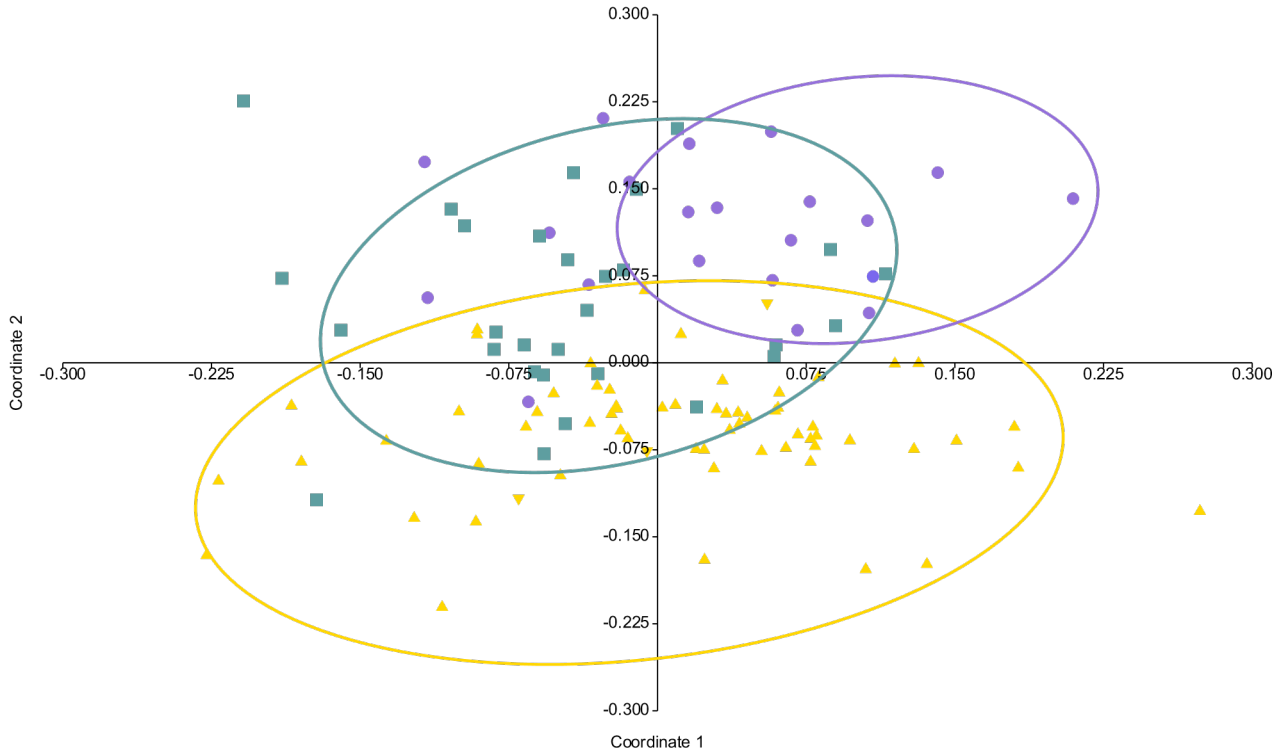


Figure 2. Non-metric multidimensional scaling (NMDS) analysis of 116 herbarium specimens of *R. baurii* varieties based on 23 morphological traits using the Gower similarity coefficient. PC axes 1 and 2 and are shown and explain 65% of the variation. PC 1 is usually heavily influenced by size measurements (i.e. leaf length and width) and including PC 1 can lead to skewed results, therefore PC 2 and PC 3 are shown in the main text.

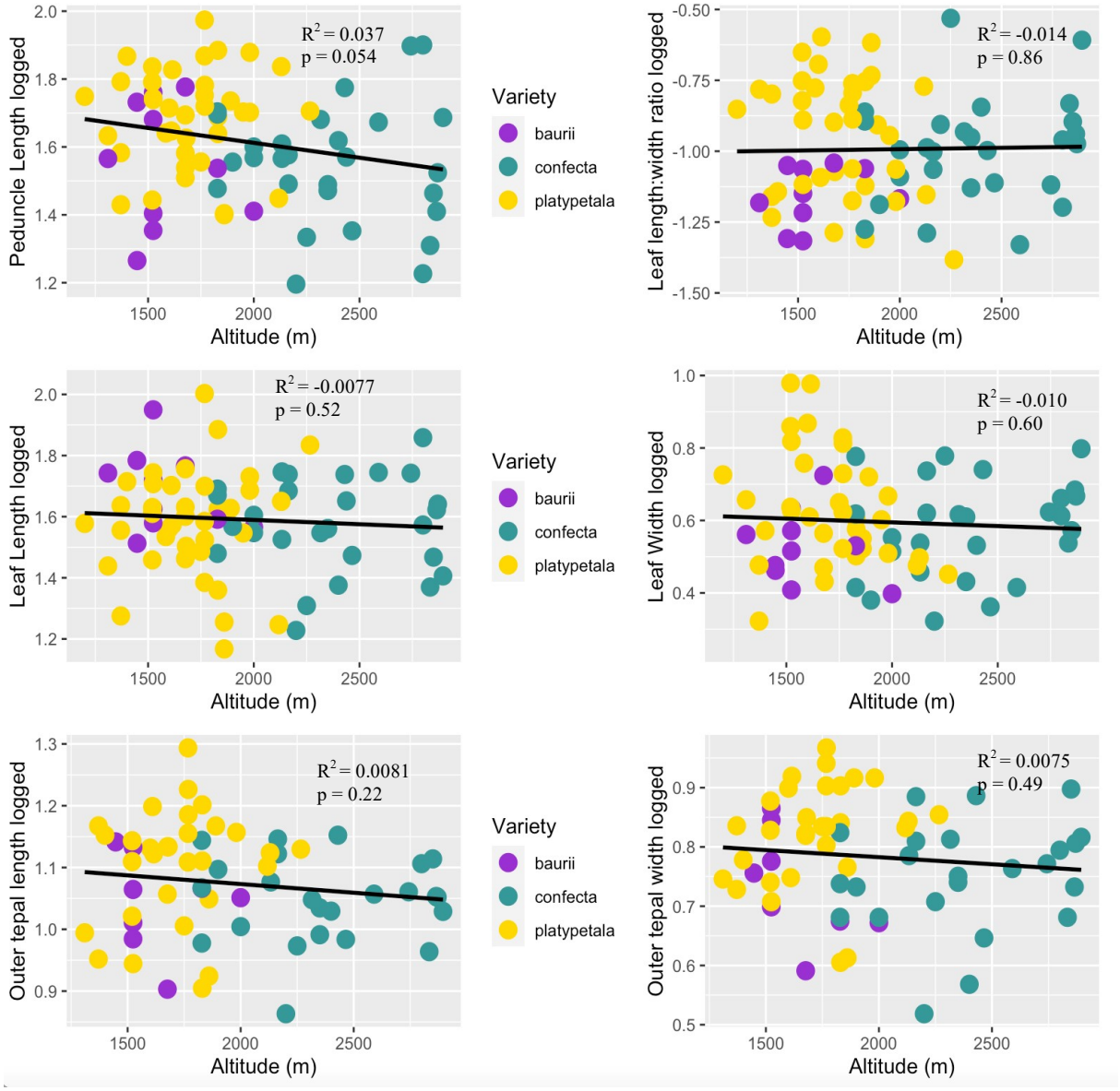


Figure 3. Correlation between the size of selected characters and altitude among *R. baurii* varieties. *Rhodohypoxis baurii* var. *platypetala* occurs mainly at low altitudes (< 2200m a.s.l) and *R. baurii* var. *confecta* occurs mainly at high altitudes (2200-3000m a.s.l).

Table 4: Relative mean fluorescence (RMF) of sample, relative mean fluorescence of standard, relative DNA content, coefficient of variation percentage (CV%) of sample and ploidy of 147 individuals of *R. baurii* var. *baurii* varieties across 9 populations. See Figure 4 below for example of flow cytometric histograms of DAPI-stained nuclei obtained.

Individual Short ID	Population	Taxon	standard RMF	sample RMF	CV%	Relative DNA content	Ploidy
b1.1	Hebron Farm	*R. baurii* var. *baurii*	106.73	205.32	8.20	1.92	2×
b1.2	Hebron Farm	*R. baurii* var. *baurii*	96.79	186.03	5.58	1.92	2×
b1.3	Hebron Farm	*R. baurii* var. *baurii*	99.84	193.25	4.93	1.94	2×
b2.1	Hebron Farm	*R. baurii* var. *baurii*	98.96	186.31	6.69	1.88	2×
b2.2	Hebron Farm	*R. baurii* var. *baurii*	104.91	201.97	5.81	1.93	2×
b2.3	Hebron Farm	*R. baurii* var. *baurii*	109.45	207.75	5.23	1.90	2×
b3.1	Hebron Farm	*R. baurii* var. *baurii*	102.02	187.97	5.32	1.84	2×
b3.2	Hebron Farm	*R. baurii* var. *baurii*	102.76	195.72	5.34	1.90	2×
b3.3	Hebron Farm	*R. baurii* var. *baurii*	101.41	189.02	7.85	1.86	2×
b4.1	Hebron Farm	*R. baurii* var. *baurii*	107.01	189.46	4.95	1.77	2×
b4.2	Hebron Farm	*R. baurii* var. *baurii*	108.02	190.33	4.57	1.76	2×
b4.3	Hebron Farm	*R. baurii* var. *baurii*	109.04	192.45	4.32	1.76	2×
b5.1	Hebron Farm	*R. baurii* var. *baurii*	112.43	195.31	5.28	1.74	2×
b5.2	Hebron Farm	*R. baurii* var. *baurii*	105.81	275.19	5.07	2.60	3×
b5.3	Hebron Farm	*R. baurii* var. *baurii*	104.36	185.33	5.67	1.78	2×
b6.1	Hebron Farm	*R. baurii* var. *baurii*	105.86	187.76	6.24	1.77	2×
b6.2	Hebron Farm	*R. baurii* var. *baurii*	103.6	185.63	5.87	1.79	2×
b6.3	Hebron Farm	*R. baurii* var. *baurii*	109.3	192.49	4.35	1.76	2×
b7.1	Hebron Farm	*R. baurii* var. *baurii*	102.61	276.35	4.34	2.69	3×
b7.2	Hebron Farm	*R. baurii* var. *baurii*	100.91	271.21	5.24	2.69	3×
b7.3	Hebron Farm	*R. baurii* var. *baurii*	100.1	268.39	4.57	2.68	3×
b8.1	Hebron Farm	*R. baurii* var. *baurii*	105.22	375.82	4.25	3.57	4×
b8.2	Hebron Farm	*R. baurii* var. *baurii*	102.51	372.08	6.29	3.63	4×
b8.3	Hebron Farm	*R. baurii* var. *baurii*	108.82	400.82	5.89	3.68	4×
b9.1	Hebron Farm	*R. baurii* var. *baurii*	107.61	196.54	5.46	1.83	2×
b9.2	Hebron Farm	*R. baurii* var. *baurii*	107.9	193.37	5.72	1.79	2×
b9.3	Hebron Farm	*R. baurii* var. *baurii*	108.42	194.64	4.92	1.80	2×
b10.1	Hebron Farm	*R. baurii* var. *baurii*	107.62	191.28	6.00	1.78	2×
b10.2	Hebron Farm	*R. baurii* var. *baurii*	107.83	191.56	4.32	1.78	2×
b10.3	Hebron Farm	*R. baurii* var. *baurii*	107.95	190.21	4.82	1.76	2×
BH13_1	Hebron Farm	*R. baurii* var. *baurii*	106.98	189.89	4.20	1.78	2×
BH13_2	Hebron Farm	*R. baurii* var. *baurii*	106.45	190	4.70	1.78	2×
BH13_3	Hebron Farm	*R. baurii* var. *baurii*	105.87	188.19	3.90	1.78	2×
c1.1	Sentinel	*R. baurii* var. *confecta*	109.7	201.86	4.91	1.84	2×
c1.2	Sentinel	*R. baurii* var. *confecta*	106.75	186.68	6.00	1.75	2×
c1.3	Sentinel	*R. baurii* var. *confecta*	102.97	179.33	5.51	1.74	2×
c2.1	Sentinel	*R. baurii* var. *confecta*	103.66	372.77	5.13	3.60	4×
c2.2	Sentinel	*R. baurii* var. *confecta*	104.33	372.82	3.98	3.57	4×
c2.3	Sentinel	*R. baurii* var. *confecta*	107.63	385.68	3.79	3.58	4×
c3.1	Sentinel	*R. baurii* var. *confecta*	106.56	266.46	4.40	2.50	2×/3×

c3.2	Sentinel	*R. baurii* var. *confecta*	113.18	191.36	6.16	1.69	2×
c3.3	Sentinel	*R. baurii* var. *confecta*	109.56	190.2	5.51	1.74	2×
c4.1	Platberg	*R. baurii* var. *confecta*	105.71	365.62	4.52	3.46	4×
c4.2	Platberg	*R. baurii* var. *confecta*	97.26	334.92	5.03	3.44	4×
c4.3	Platberg	*R. baurii* var. *confecta*	109.85	369.43	5.07	3.36	4×
c5.1	Platberg	*R. baurii* var. *confecta*	119.41	402.27	4.18	3.37	4×
c5.2	Platberg	*R. baurii* var. *confecta*	109.26	386.54	4.26	3.54	4×
c5.3	Platberg	*R. baurii* var. *confecta*	98.94	335.28	5.05	3.39	4×
c6.1	Sentinel	*R. baurii* var. *confecta*	107.3	183.42	5.43	1.71	2×
c6.2	Sentinel	*R. baurii* var. *confecta*	106.93	186.24	4.98	1.74	2×
c6.3	Sentinel	*R. baurii* var. *confecta*	106.86	190.64	4.88	1.78	2×
c7.1	Sentinel	*R. baurii* var. *confecta*	103.89	180.1	4.30	1.73	2×
c7.2	Sentinel	*R. baurii* var. *confecta*	104.76	182.54	4.88	1.74	2×
c7.3	Sentinel	*R. baurii* var. *confecta*	106.09	183.67	5.95	1.73	2×
c8.1	Sentinel	*R. baurii* var. *confecta*	107.34	182.91	5.99	1.70	2×
c8.2	Sentinel	*R. baurii* var. *confecta*	99.64	167.86	4.64	1.68	2×
c8.3	Sentinel	*R. baurii* var. *confecta*	108.97	178.68	4.60	1.64	2×
c9.1	Platberg	*R. baurii* var. *confecta*	90.89	341.21	6.17	3.75	4×
c9.2	Platberg	*R. baurii* var. *confecta*	97.25	365.42	4.47	3.76	4×
c9.3	Platberg	*R. baurii* var. *confecta*	97.81	357.02	3.50	3.65	4×
c10.1	Sentinel	*R. baurii* var. *confecta*	116.53	209.71	5.78	1.80	2×
c10.2	Sentinel	*R. baurii* var. *confecta*	109.5	193.45	5.30	1.77	2×
c10.3	Sentinel	*R. baurii* var. *confecta*	133.42	235.21	4.24	1.76	2×
p1.1	Karkloof	*R. baurii* var. *platypetala*	104.51	389.31	4.56	3.73	4×
p1.2	Karkloof	*R. baurii* var. *platypetala*	108.83	389.33	4.98	3.58	4×
p1.3	Karkloof	*R. baurii* var. *platypetala*	103.86	368.38	3.93	3.55	4×
p2.1	Hebron Farm	*R. baurii* var. *platypetala*	112.31	404.9	4.09	3.61	4×
p2.2	Hebron Farm	*R. baurii* var. *platypetala*	116.23	208.11	5.08	1.79	2×
p2.3	Hebron Farm	*R. baurii* var. *platypetala*	108.22	383.64	5.49	3.55	4×
p3.1	Sunset Farm	*R. baurii* var. *platypetala*	117.73	207.07	4.63	1.76	2×
p3.2	Sunset Farm	*R. baurii* var. *platypetala*	108.47	186.17	4.65	1.72	2×
p3.3	Sunset Farm	*R. baurii* var. *platypetala*	110.72	197.4	5.06	1.78	2×
p4.1	Karkloof	*R. baurii* var. *platypetala*	100.93	178.7	5.17	1.77	2×
p4.2	Karkloof	*R. baurii* var. *platypetala*	104.92	183.21	5.57	1.75	2×
p4.3	Karkloof	*R. baurii* var. *platypetala*	105.03	183.33	4.56	1.75	2×
p5.1	Sunset Farm	*R. baurii* var. *platypetala*	109.75	287.44	4.46	2.62	3×
p5.2	Sunset Farm	*R. baurii* var. *platypetala*	120.55	316.82	3.26	2.63	3×
p5.3	Sunset Farm	*R. baurii* var. *platypetala*	110.09	293.78	4.52	2.67	3×
p6.1	Sunset Farm	*R. baurii* var. *platypetala*	109.05	192.19	4.96	1.76	4×
p6.2	Sunset Farm	*R. baurii* var. *platypetala*	105.37	186.18	4.35	1.77	4×
p6.3	Sunset Farm	*R. baurii* var. *platypetala*	112.29	200.91	4.73	1.79	4×
p7.1	Hebron Farm	*R. baurii* var. *platypetala*	106.38	364.64	4.81	3.43	4×
p7.2	Hebron Farm	*R. baurii* var. *platypetala*	113.56	389.58	3.95	3.43	4×
p7.3	Hebron Farm	*R. baurii* var. *platypetala*	109.24	384.78	3.61	3.52	4×
p8.1	Hebron Farm	*R. baurii* var. *platypetala*	101.85	360.82	3.59	3.54	4×
p8.2	Hebron Farm	*R. baurii* var. *platypetala*	103.98	361.56	3.22	3.48	4×

p8.3	Hebron Farm	*R. baurii* var. *platypetala*	110.68	380.68	3.24	3.44	4×
p9.1	Sunset Farm	*R. baurii* var. *platypetala*	104.73	182.09	5.76	1.74	2×
p9.2	Sunset Farm	*R. baurii* var. *platypetala*	96.44	174.78	4.52	1.81	2×
p9.3	Sunset Farm	*R. baurii* var. *platypetala*	100.12	178.43	5.30	1.78	2×
p10.1	Golden Gate	*R. baurii* var. *platypetala*	108.24	278.05	3.63	2.57	3×
p10.2	Golden Gate	*R. baurii* var. *platypetala*	105.6	367.21	4.04	3.48	3×
p10.2	Golden Gate	*R. baurii* var. *platypetala*	106.78	371.11	4.45	3.48	3×
p10.3	Golden Gate	*R. baurii* var. *platypetala*	108.01	190.69	5.81	1.77	3×
VB_b1	Vrederus	*R. baurii* var. *baurii*	109.11	375.22	3.48	3.44	4×
VB_b2	Vrederus	*R. baurii* var. *baurii*	109.95	377.2	3.45	3.43	4×
VB_b3	Vrederus	*R. baurii* var. *baurii*	101.04	354.43	5.79	3.51	4×
VB_b4	Vrederus	*R. baurii* var. *baurii*	100.23	354.67	4.87	3.54	4×
VB_b5	Vrederus	*R. baurii* var. *baurii*	102.45	356.2	4.92	3.48	4×
VB_b6	Vrederus	*R. baurii* var. *baurii*	104.17	355.75	5.15	3.42	4×
VB_b7	Vrederus	*R. baurii* var. *baurii*	103.45	354.67	4.32	3.43	4×
VB_b8	Vrederus	*R. baurii* var. *baurii*	102.76	355.98	4.67	3.46	4×
VB_b9	Vrederus	*R. baurii* var. *baurii*	102.87	353.98	5.12	3.44	4×
VB_b10	Vrederus	*R. baurii* var. *baurii*	101.89	352.98	4.89	3.46	4×
VB_b11	Vrederus	*R. baurii* var. *baurii*	104.12	362.87	4.91	3.49	4×
VB_b12	Vrederus	*R. baurii* var. *baurii*	105.72	365.23	4.21	3.45	4×
VB_b13	Vrederus	*R. baurii* var. *baurii*	103.47	355.76	4.35	3.44	4×
VB_b14	Vrederus	*R. baurii* var. *baurii*	104.25	356.78	4.76	3.42	4×
VB_b15	Vrederus	*R. baurii* var. *baurii*	100.78	352.11	5.12	3.49	4×
VB_b16	Vrederus	*R. baurii* var. *baurii*	103.23	354.67	5.23	3.44	4×
VB_b17	Vrederus	*R. baurii* var. *baurii*	101.45	352.16	5.30	3.47	4×
VB_b18	Vrederus	*R. baurii* var. *baurii*	102.87	353.33	5.00	3.43	4×
VB_b19	Vrederus	*R. baurii* var. *baurii*	95.97	341.18	4.65	3.56	4×
VB_b20	Vrederus	*R. baurii* var. *baurii*	97.87	350.23	4.87	3.58	4×
VB_b21	Vrederus	*R. baurii* var. *baurii*	96.66	352.22	4.76	3.64	4×
VB_b22	Vrederus	*R. baurii* var. *baurii*	103.35	357.95	5.10	3.46	4×
VB_b24	Vrederus	*R. baurii* var. *baurii*	109.28	374.64	5.85	3.43	4×
NN_b1	Naudes Nek	*R. baurii* var. *baurii*	102.76	362.1	4.89	3.52	4×
NN_b2	Naudes Nek	*R. baurii* var. *baurii*	103.66	353.57	5.38	3.41	4×
NN_b3	Naudes Nek	*R. baurii* var. *baurii*	97.05	377.2	6.74	3.89	4×
NN_b4	Naudes Nek	*R. baurii* var. *baurii*	102.45	355.75	3.48	3.47	4×
NN_b5	Naudes Nek	*R. baurii* var. *baurii*	101.89	352.98	5.79	3.46	4×
NN_b6	Naudes Nek	*R. baurii* var. *baurii*	104.25	351.18	5.12	3.37	4×
NN_b9	Naudes Nek	*R. baurii* var. *baurii*	101.45	362.1	4.76	3.57	4×
NN_b10	Naudes Nek	*R. baurii* var. *baurii*	96.66	351.2	4.76	3.63	4×
NN_b11	Naudes Nek	*R. baurii* var. *baurii*	109.28	378.2	4.89	3.46	4×
NN_b12	Naudes Nek	*R. baurii* var. *baurii*	108.65	367.2	5.38	3.38	4×
NN_b13	Naudes Nek	*R. baurii* var. *baurii*	107.39	389.1	5.57	3.62	4×
NN_b15	Naudes Nek	*R. baurii* var. *baurii*	103.22	387.33	4.96	3.75	4×
NN_b16	Naudes Nek	*R. baurii* var. *baurii*	97.67	365.43	4.52	3.74	4×
NN_b18	Naudes Nek	*R. baurii* var. *baurii*	98.76	351.87	3.63	3.56	4×
NN_b20	Naudes Nek	*R. baurii* var. *baurii*	99.43	361.22	4.45	3.63	4×

NN_b21	Naudes Nek	*R. baurii* var. *baurii*	101.34	372.77	3.48	3.68	4×
NN_b22	Naudes Nek	*R. baurii* var. *baurii*	104.34	375.4	4.87	3.60	4×
NN_b23	Naudes Nek	*R. baurii* var. *baurii*	103.24	374.44	4.89	3.63	4×
NN_b24	Naudes Nek	*R. baurii* var. *baurii*	104.38	378.2	3.80	3.62	4×
NN_b27	Naudes Nek	*R. baurii* var. *baurii*	105.45	385.4	3.80	3.65	4×
NN_b28	Naudes Nek	*R. baurii* var. *baurii*	114.57	397.11	4.66	3.47	4×
T_b1	Tenahead Lodge	*R. baurii* var. *baurii*	101.5	377.01	4.20	3.71	4×
T_b2	Tenahead Lodge	*R. baurii* var. *baurii*	100	378.21	5.00	3.78	4×
T_b3	Tenahead Lodge	*R. baurii* var. *baurii*	94.38	357.28	4.76	3.79	4×
T_b4	Tenahead Lodge	*R. baurii* var. *baurii*	103.29	385.15	6.56	3.73	4×
T_b5	Tenahead Lodge	*R. baurii* var. *baurii*	109.23	413.81	4.91	3.79	4×
T_b6	Tenahead Lodge	*R. baurii* var. *baurii*	101.56	391.21	4.78	3.85	4×
T_b7	Tenahead Lodge	*R. baurii* var. *baurii*	99.99	387.22	4.99	3.87	4×
T_b8	Tenahead Lodge	*R. baurii* var. *baurii*	100.61	395.61	3.45	3.93	4×
T_b9	Tenahead Lodge	*R. baurii* var. *baurii*	106.78	390.39	5.48	3.66	4×

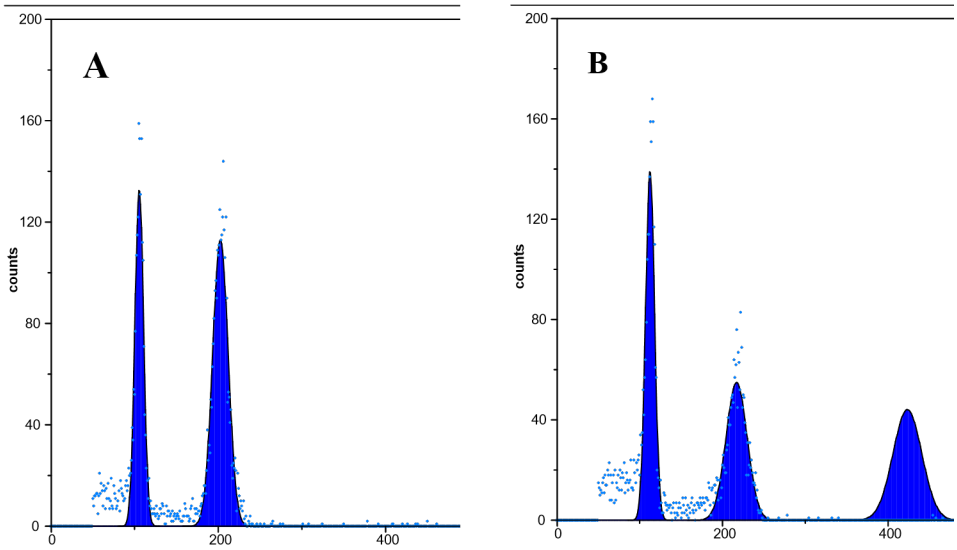


Figure 4. Flow cytometric histograms of DAPI-stained nuclei obtained from analyses of *Rhodohypoxis* individuals using *Oxalis articulata* as the standard. Flow cytometric histograms showing the peak profile of **A:** diploid individual *R. baurii* var. *baurii* from Hebron Farm (17BH17) and **B:** tetraploid individual *R. baurii* var. *platypetala* Hebron Farm (15PH2).

Table 5. 12 microsatellite markers used and the number of polymorphic loci.

Microsatellite marker ID	**no. polymorphic loci**
AG_Rba_444	13
AG_Rba_165	15
AG_Rba_448	23
AG_Rba_307	36
AG_Rba_041	12
AG_Rba_158	14

AG_Rba_304	9
AG_Rba_206	18
AG_Rba_290	19
AG_Rba_473	21
AG_Rba_489	9
AG_Rba_182	32
Total polymorphic loci:	221

Table 6. Nei's genetic differentiation (G'st fixation index) for the 11 sampled *Rhodohypoxis baurii* populations. GG = Golden Gate; PB = Platberg; Sent = Sentinel; HF = Hebron Farm, SC = Sani Chalet; SBT = Sethlabethebe,;SF = Sunset Farm, TL = Tenahead Lodge; NN =. Naudes Nek, VB = Vrederus; KF = Karkloof.

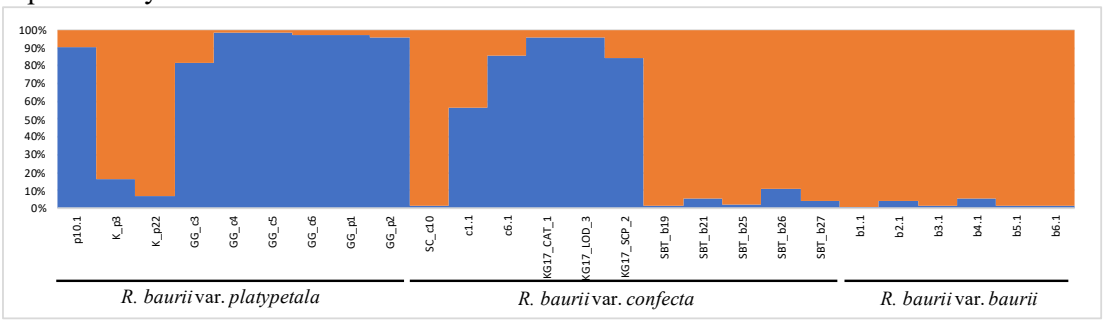
Obs.	GG	PB	Sent	HF	SC	SBT	SF	TL	NN	VB	KF
GG	0										
PB	0.216999	0									
Sent	0.123478	0.126245	0								
HF	0.216579	0.213488	0.149407	0							
SC	0.20592	0.180305	0.116823	0.075995	0						
SBT	0.211666	0.182616	0.138597	0.062931	0.064869	0					
SF	0.178823	0.17067	0.115046	0.080006	0.058988	0.054403	0				
TL	0.211756	0.181027	0.188726	0.098142	0.140279	0.092655	0.123262	0			
NN	0.15928	0.140094	0.104685	0.142759	0.10092	0.11571	0.133646	0.103739	0		
VB	0.196032	0.186449	0.16707	0.098142	0.099114	0.082505	0.107282	0.060881	0.081194	0	
KF	0.20593	0.178993	0.1207	0.127015	0.078089	0.094743	0.070601	0.16246	0.10769	0.155902	0

Table 7. Nei's genetic differentiation (G'st fixation index) for the different ploidy levels in *Rhodohypoxis baurii.*

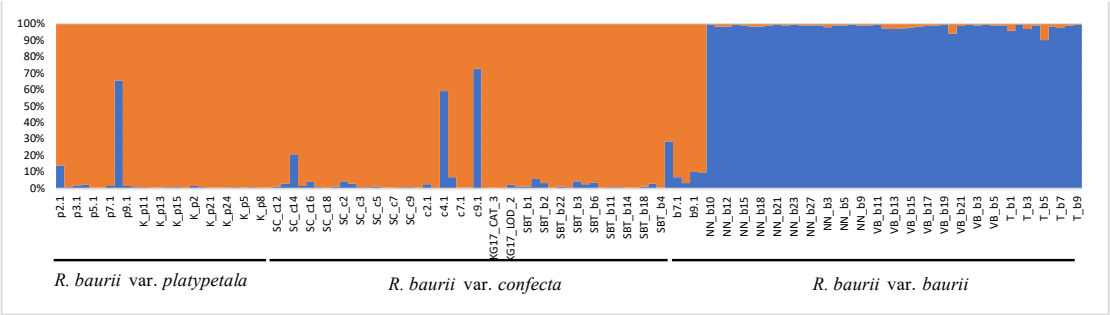
Nei's	Diploid	Triploid	Tetraploid
Diploid	0	0.102	0.158
Triploid	0.102	0	0.061
Tetraploid	0.158	0.061	0

K=2

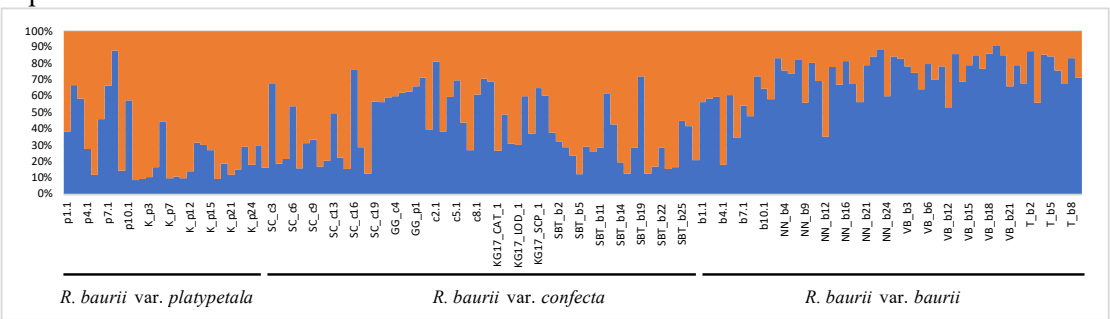
Diploids only



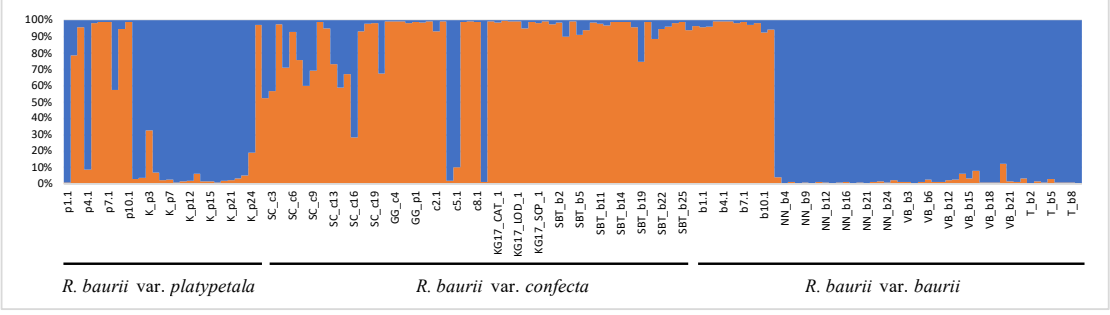
Polyploids only



Diploidised

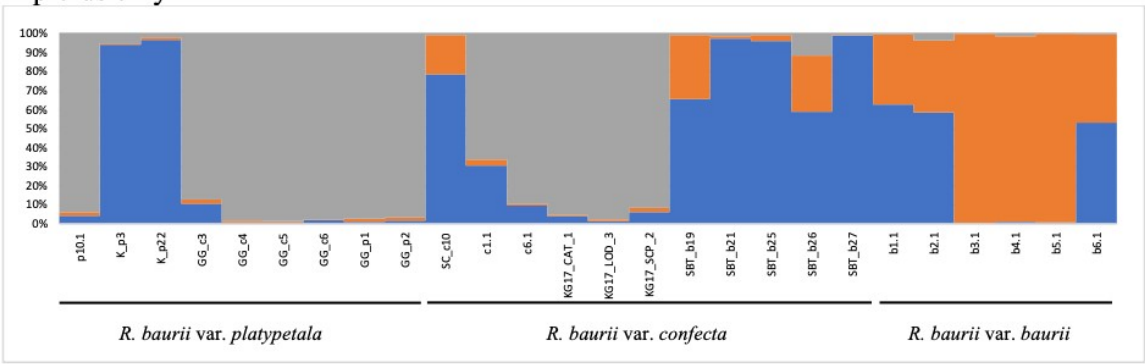


Polyploidized



K=3

Diploids only



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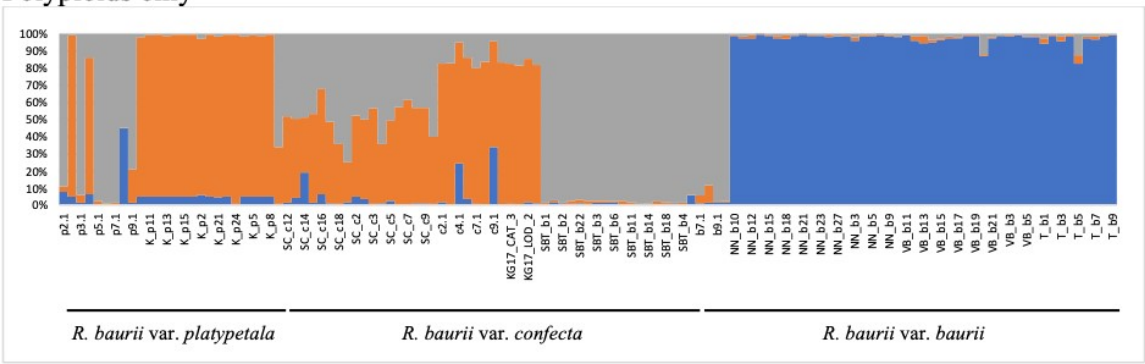

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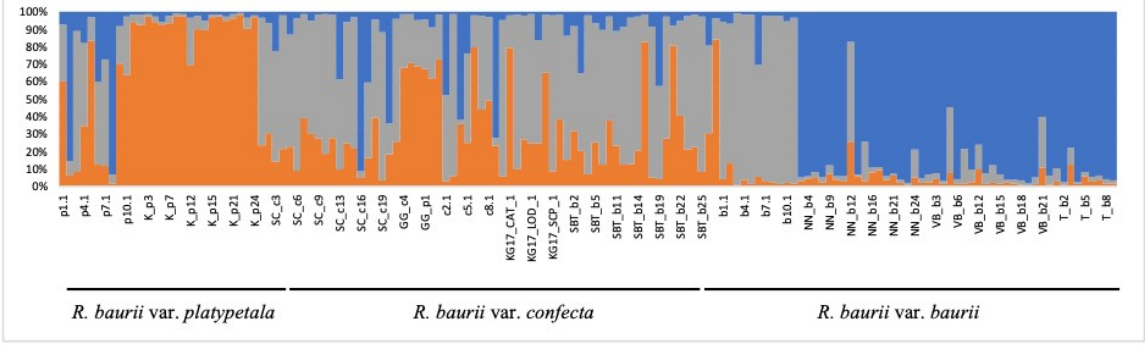

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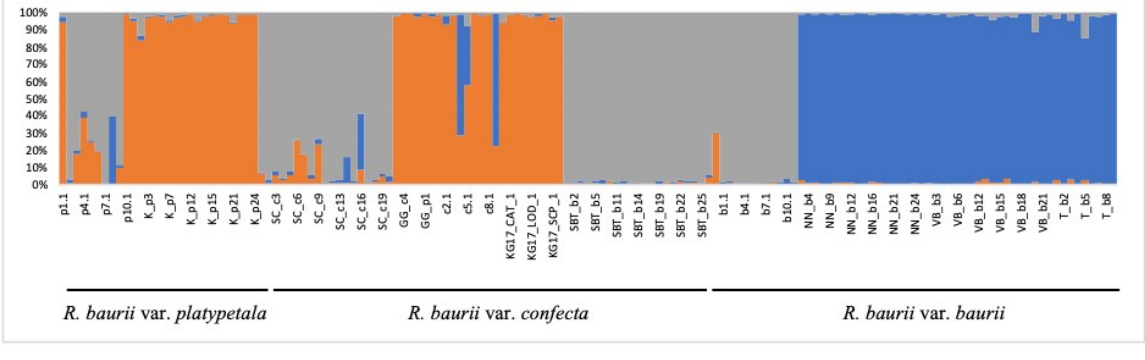
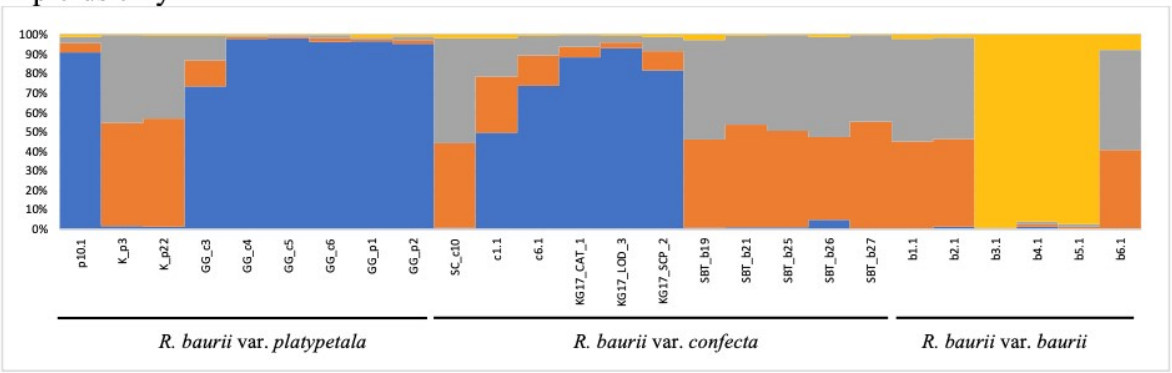
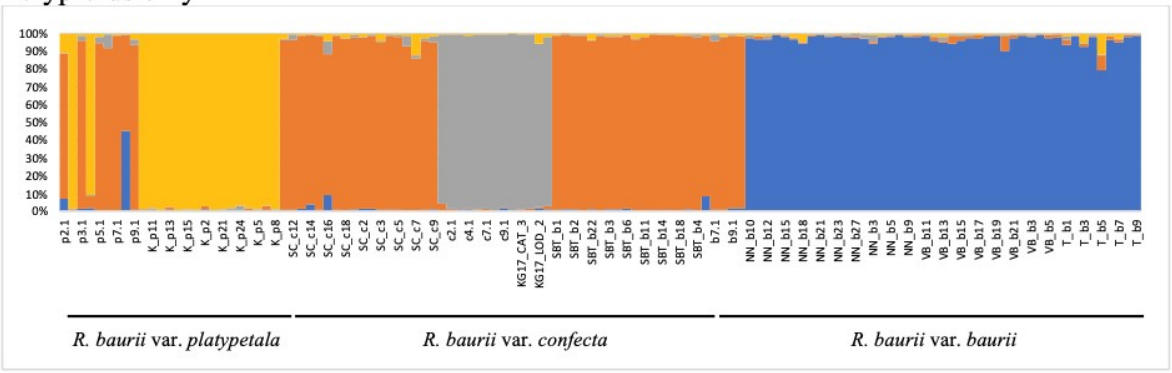


K=4

Diploids only



Polyploids only



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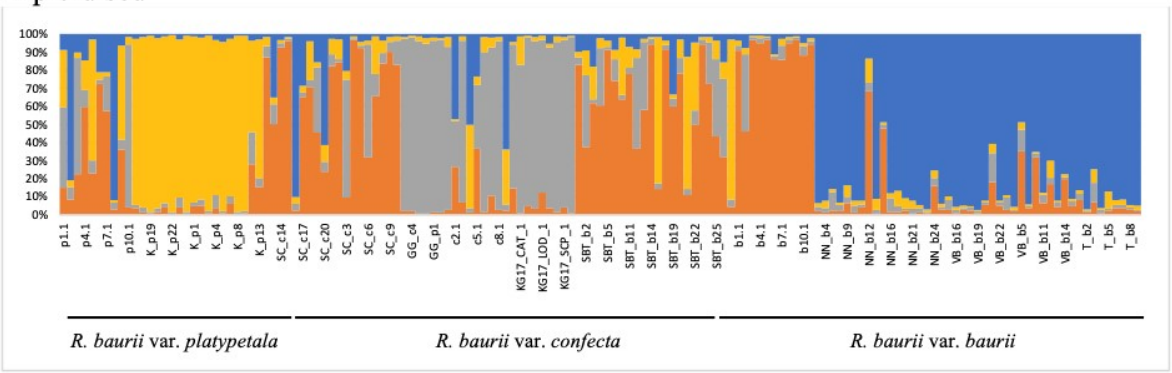

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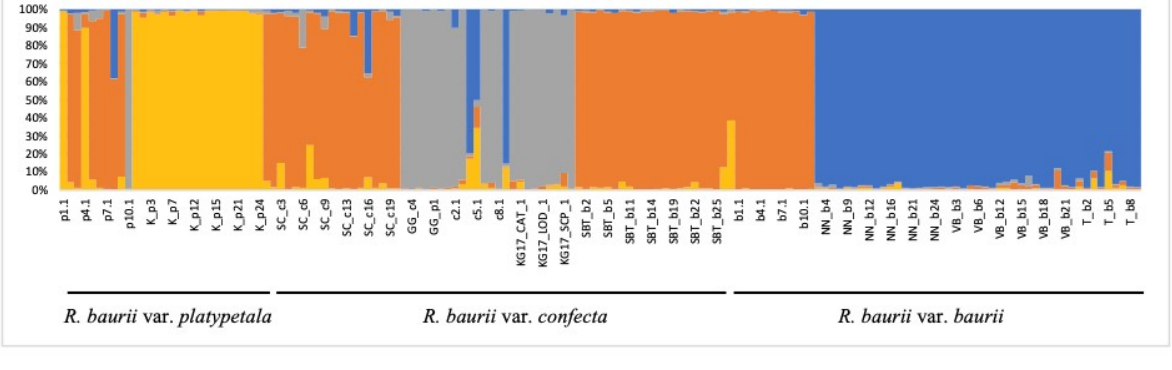
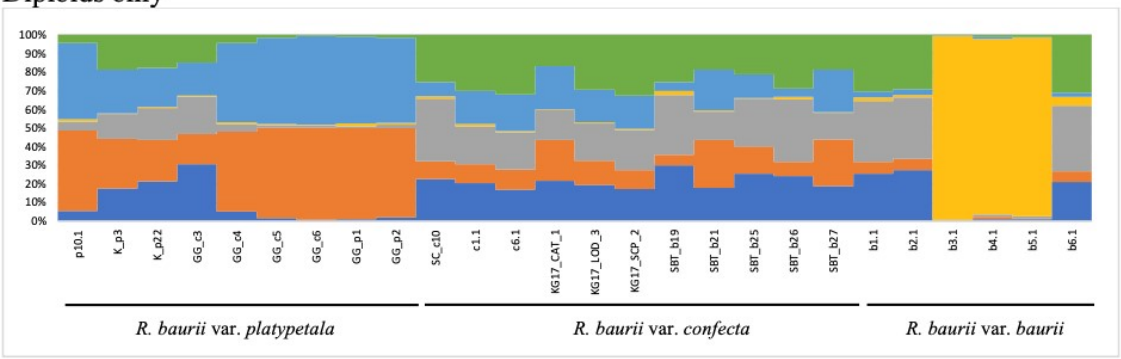
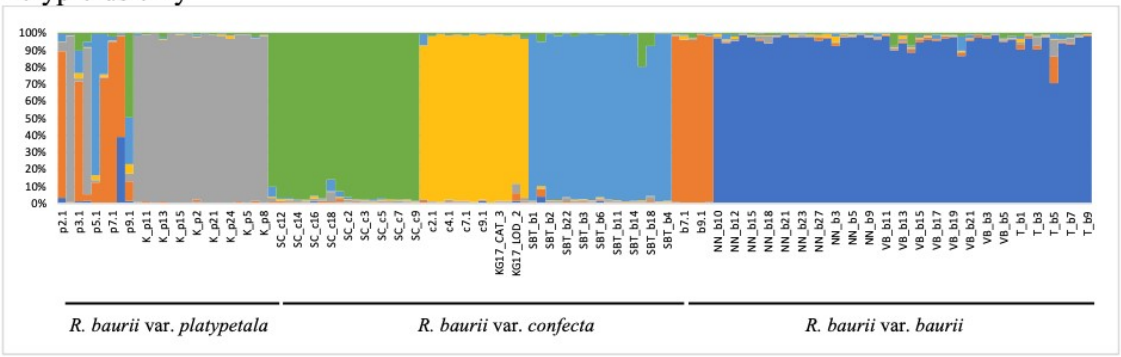


K= 6

Diploids only



Polyploids only



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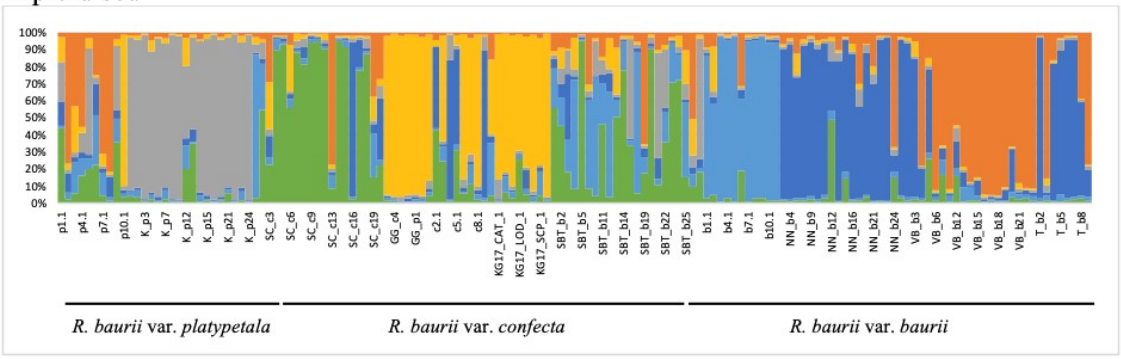
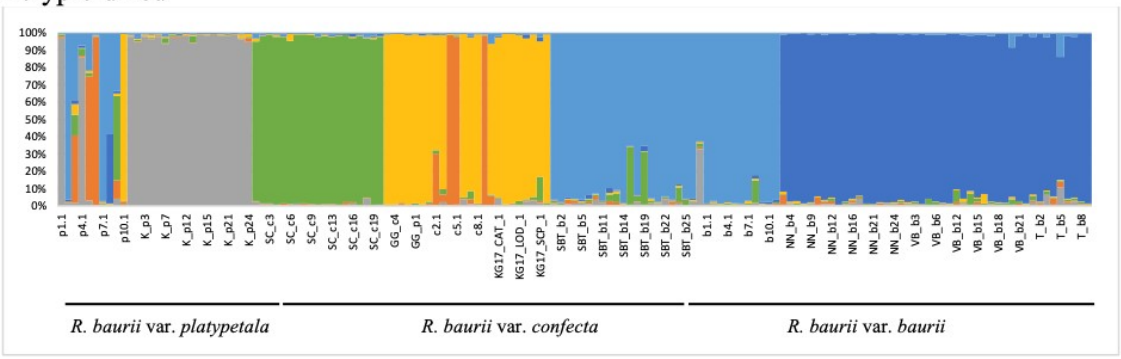
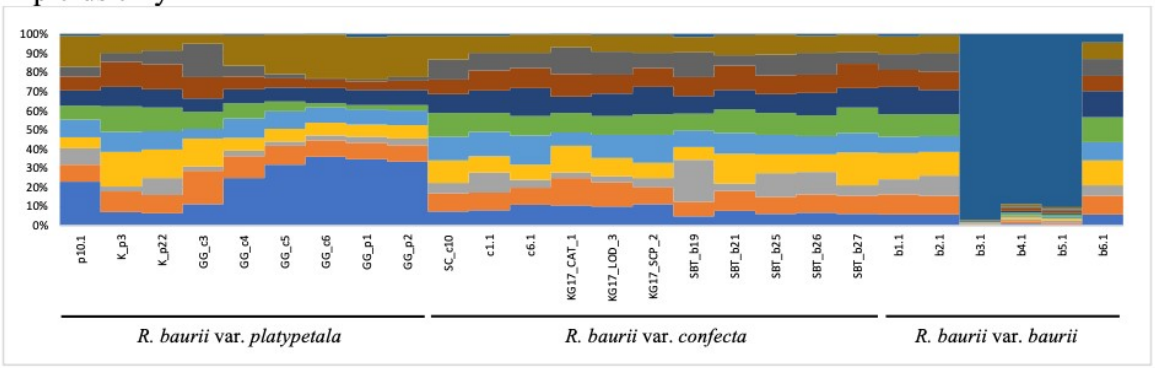


Polyploidized



K =11

Diploids only



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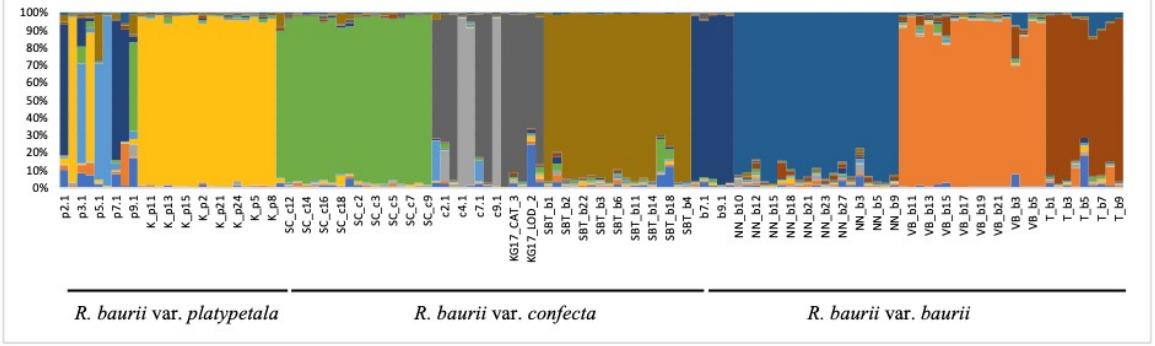

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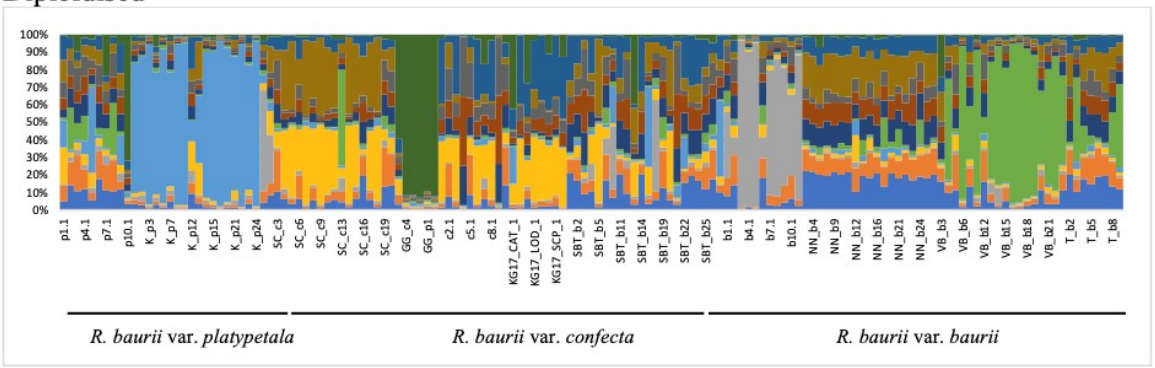

Polyploidized

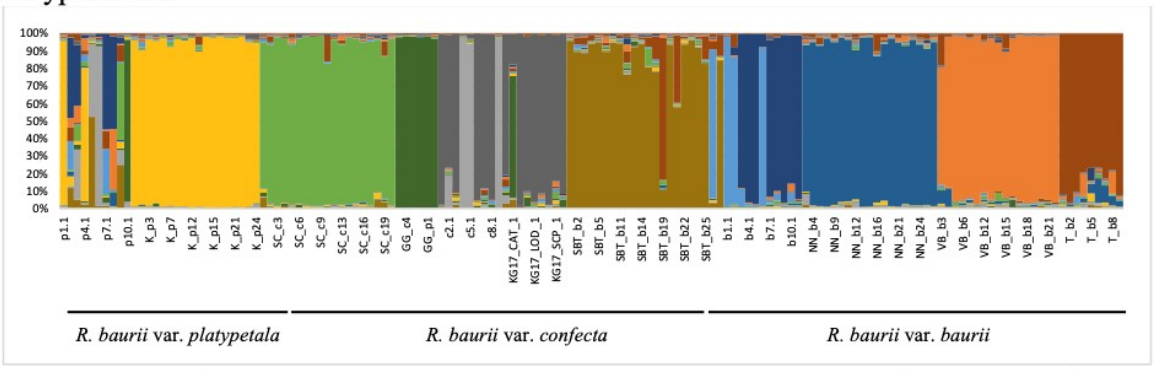

Figure 5. Genetic structure graphs of *R. baurii* for K= 2, K = 3, K = 6 and K = 11 showing the genetic structure of A: diploids only, B: Polyploids only, C: Diploidized data and D: polyploidized data.

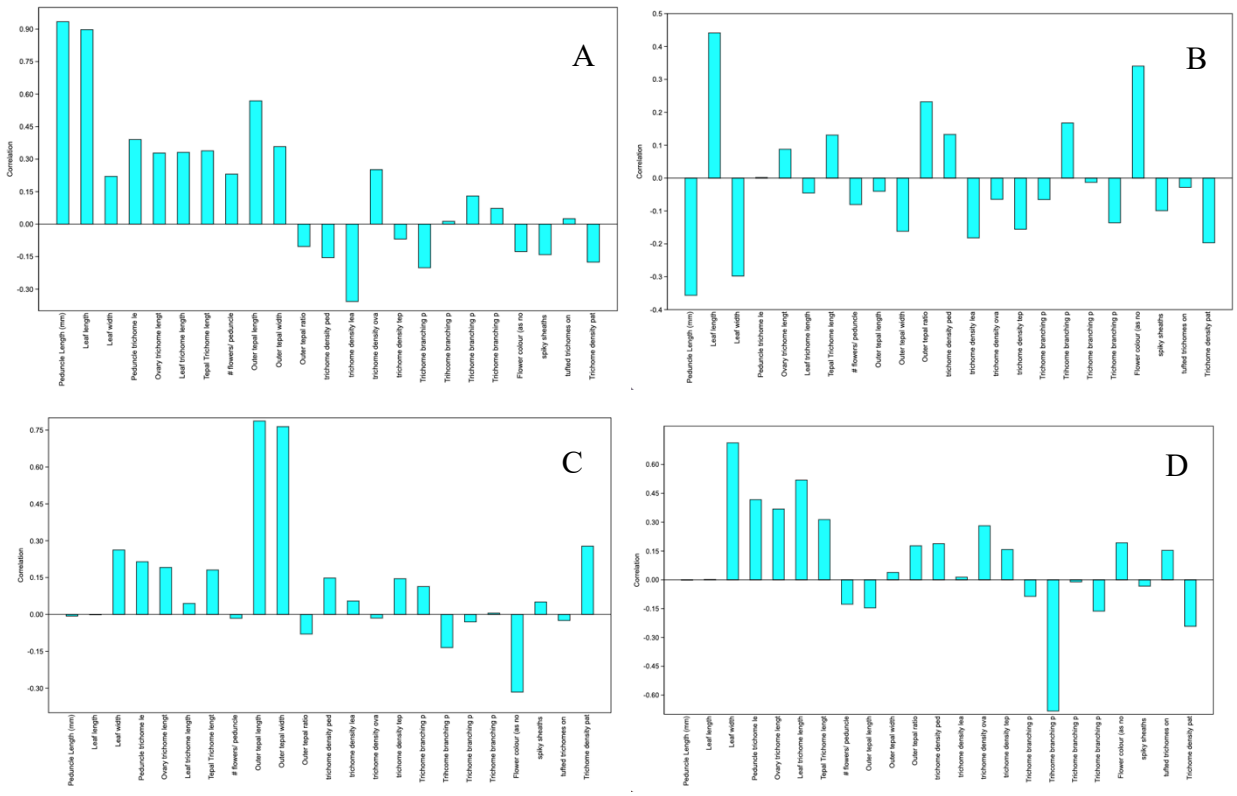

Figure 6: Contributions (loadings) based on a PCA analysis of each morphological character measured for A: Principal component 1 (PC1), B: Principal component 2 (PC2), C: Principal component 3 (PC3) and D: Principal component 4 (PC4).