Postzygotic reproductive isolation among *Rhodohypoxis baurii* varieties and *Rhodohypoxis milloides* (Hypoxidaceae).

Attang Nqobile Biyela

736157

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

December 2020, Johannesburg, South Africa

DECLARATION

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

(Attang, Nqobile Biyela)

15 December, 2020

Supervisors:

Dr. Kelsey Glennon (University of the Witwatersrand)

Prof. Glynis Cron (University of the Witwatersrand)

DEDICATION

This thesis is dedicated to my parents, Mr. Sibongakonke Biyela and Mrs. Mpho Biyela, who have been nothing but supportive of all my dreams and aspirations in life. Ngiyabonga boMenziwa.

ACKNOWLEDGEMNTS

I would like to express my gratitude to my supervisors Dr. K.L Glennon and Prof. G.V Goodman-Cron. Thank you for the kindness, patience, and dedication that you have both shown to me for the duration of this degree. To Dr. Glennon, thank you for always believing in me, and for always trying to get the best out of me at all times. You have been, and will continue to be a great inspiration to me in all spheres of life.

I would also like to express my sincerest gratitude to my friends and family for their continuous support. To Sidelisiwe Msane, Kgalalelo Setshedi, Karabo Mokeona, Gugu Mabuza, and Reitumetse Sethaba, thank you ladies for being a formidable support system. A special thank you to my dearest friend, Rufaro Samanga. Thank you for always checking up on me, even in the midst of your own battles. Thank you to Dr. Samalesu Mayonde for your kindness and patience throughout the process of teaching me molecular techniques.

I would like to acknowledge the financial support from the National Research Foundation (NRF). I would also like to thank the members of my committee for their valuable input towards my MSc.

LIST OF FIGURES

Figure 7. Five possible genetic clusters were assessed using Structure and Structure Harvester. A Delta K value of two indicates that there are two genetic clusters represented in the dataset.

Figure 8. Example of two hybrids that resulted from the reciprocal cross between parents. **a**) A hybrid of *R. milloides* from Sunset Farm and *R. baurii* var. p*latypetala* from Hebron Farm, with *R. baurii* var. *platypetala* as the maternal plant (PH3xSM12. **b**) A hybrid of *R. milloides* and *R. baurii* var. *platypetala* from Hebron Farm, with *R. milloides* from Sunset Farm as the maternal plant (SM12xPH3).

LIST OF TABLES

Table 7a. Ploidies for *R. baurii* varieties and *R. milloides*.

Table 7b. Ploidy levels and genome sizes for *Rhodohypoxis* hybrids in the greenhouse. SM = *R. milloides* from Sunset Farm, BH = *R. baurii* var. *baurii* from Hebron Farm, PH = *R. baurii* var. *platypetala* from Hebron Farm**.**

TABLE OF CONTENTS

ABSTRACT

The biodiversity we see on earth is generated through the process of speciation. Understanding how species form is central to evolutionary biology, as species are the basic units of evolutionary biology. In order to define species, biologists use species concepts as frameworks to test hypotheses around species characterization. In this study, the Biological Species Concept was used because it emphasizes that reproductive isolation occurs through the accumulation of reproductive barriers that reduce gene flow between closely related taxa. Prezygotic reproductive barriers occur before fertilization, and include habitat and pollinator isolation. Postzygotic barriers occur after fertilization and mainly manifest in hybrids through intrinsic or as extrinsic hybrid inviabilities. Postzygotic barriers are detectable through hybrids, hybridization is an important process through which reproductive isolation can be studied. Hybridization is also important inevolutionary trajectories of species because it has the potential to lead to speciation. In this study, the aim was to test for the presence of postzygotic barriers among the three varieties of *Rhodohypoxis baurii* and *R. milloides* from two different localities. Data obtained from controlled greenhouse crosses showed successful interbreeding between the varieties, as well as between the varieties and *R. milloides.* Seeds germinated from these controlled crosses were used to test hybrid viability through germination success. The analysis of gene flow and population structure between the four taxa was done using amplified fragment polymorphisms (AFLPs). Flow cytometry was used to determine ploidy and found that *R. baurii* var. *baurii* and *R. baurii* var. *confecta* to be diploid, and *R. baurii* var. *platypetala* and *R. milloides* to be tetraploid. Ploidy was not found to affect hybrid or seed viability. Results also show that there are few prezygotic barriers between the three *R. baurii* varieties and the three *R. baurii* varieties and *R. milloides*. Both the Hebron Farm and Sunset Farm populations are highly admixed due to ongoing introgression. Postzygotic isolation between *Rhodohypoxis baurii* varieties and *R. milloides* does not manifest as seed inviability, but may be seen in later hybrid generations in the form

of extrinsic hybrid inviability, or as pollen inviability. The main findings imply that *Rhodohypoxis baurii* varieties cannot be elevated to specific level, and that hybridization and habitat isolation have defining roles in the evolutionary trajectory of the species.

CHAPTER 1: LITERATURE REVIEW

1. Literature review

Understanding how species form is central to evolutionary biology, as species are the basic units of evolutionary biology, ecology, taxonomy, and conservation biology (Barton, 2001; De Queiroz, 2005). Charles Darwin essentially laid the foundation for the field of evolutionary biology through his publication of *On the Origin of Species,* where he questioned how species are formed (Darwin, 1859, Grant and Grant, 1997). Darwin also introduced the concepts of evolution by natural selection, descent through modification, and that the characteristics of organisms are maintained from generation to generation. What was lacking in Darwin's understanding were the mechanisms through which characteristics were retained. In the late 1930's, Theodosius Dobzhansky built on Darwin's work, and introduced the idea of genetic changes as part of the evolutionary process in his book, *Genetics and the Origin of Species* (Dobzhansky, 1937). In this book, Dobzhansky further developed our understanding of how species form by introducing the concept of isolating mechanisms that develop during the process of divergence. These advances have ultimately influenced how biologists study speciation today.

Defining an evolutionary lineage below the species level is equally, if not more challenging, particularly when considering plants due to their high frequency of hybridization. Botanists have struggled with the challenge of delineating species (Sites and Marshall, 2003; Wiens, 2007) – such a challenge is more complicated when considering that there are multiple ways in which the speciation process can be viewed. For instance, speciation may be viewed as being geographical, where species either develop in allopatry, sympatry, parapatry, or peripatry (Mayr, 1942; Smith, 1966). Speciation can also be viewed as being ecological or non-ecological; speciation is viewed as being ecological when external forces impose a selection pressure that results in reproductive isolation (Sobel *et al*, 2010). If one views speciation as a set of stages that a group of individuals collectively pass through,

then delineating entities below the species level may help with better understanding how lineages diverge, and at what stage divergence is likely complete (Hey *et al.*, 2003). The collective change in phenotypes across environmental gradients applies a selection pressure that may or may not facilitate a type of reproductive isolation. One then explores the strength of postzygotic isolation mechanisms versus prezygotic isolation mechanismsto determine the fluidity of ecotypes (*sensu* Lowry, 2012) and the likelihood that these groups may eventually become reproductively isolated.

There has been much debate around defining species amongst evolutionary biologists. To try and solve the problem of defining what species are, and how they arise (termed the "species problem"), biologists have developed multiple species concepts. A major concern around these multiple species concepts is that they are incompatible with each other, and that they lead to different conclusions regarding species delimitation and the overall number of species in general (de Queiroz, 2007). One of the reasons for this is that different groups of biologist may define species in accordance to what is of interest to their particular field of study. Most species concepts have been developed by zoologists, who have used species whose reproductive isolation is somewhat "complete", and so delimitation has not been an issue. The real challenge does however arise with the delimitation and defining of species outside of the animal kingdom (Mishler and Donoghue, 1982), particularly with plants because of their ability to reproduce across species barriers.

Species concepts can be mechanistic, historical, or morphological (Levin, 2000). Morphological species concepts place the emphasis on defining phenotypic characteristics. For example, the Phenetic Species Concept (Michener, 1970) defines species as organisms that are phenotypically similar to each other through the use of as many characters as possible. Historical species concepts such as the Evolutionary Species Concept (Simpson, 1961; Wiley, 1981) or the Phylogenetic Species Concept (Nixon and Wheeler, 1990)

emphasize evolutionary processes (Levin, 2000). The Biological Species Concept (BSC; Mayr, 1942), is a mechanistic species concept that defines species based on the presence of reproductive isolation between populations. Species concepts provide a framework to test hypotheses around species diversification. For example, scientists often use the BSC when studying mechanisms of reproductive isolation because it emphasises that gene flow between species is reduced by barriers to reproduction (e.g. Dobzhansky, 1937; Mayr, 1942; Coyne and Orr, 1989; Ramsey *et al.*, 2003; Kay, 2006; Lowry *et al.*, 2008).

Reproductive isolation is the result of a gradual accumulation of phenotypic and genotypic differences between closely related species (Ramsey *et al.*, 2003; Lowry *et al.*, 2008). Such differences may eventually lead to species divergence (Rieseberg and Willis 2007; Sobel *et al.*, 2010). Understanding the contribution of reproductive barriers to speciation and the role they play in the persistence of species is key to understanding how species evolve (Ramsey *et al.*, 2003; Lowry *et al.*, 2008; Dell'Olivio *et al.*, 2011), and to understanding patterns of species richness. Better understanding of reproductive barriers can aid in elucidating the conditions under which the various barriers arise, allowing us to postulate past conditions that contributed to the present species richness. The emergence of reproductive barriers is thought to be sequential, with the total strength of reproductive barriers hypothesized to be directly related to the divergence time of taxa (Coyne and Orr, 1989). Early acting barriers are thought to be the most instrumental in reducing gene flow between populations, because of the correlation between barrier accumulation and divergence time (Coyne and Orr, 2004, Lowry *et al.*, 2008).

Consequently, studies pertaining to reproductive isolation often aim to quantify the strengths of isolating barriers in the speciation process (Ramsey *et al.*, 2003; Coyne and Orr, 2004; Rundle and Nosil, 2005; Kay, 2006; Lowry *et al.*, 2008). Coyne and Orr's (1989) foundational work on reproductive isolation began quantifying the strength of reproductive

isolation when they sought to investigate the time course of speciation in six *Drosophila* Fallén species (*Drosophila pseudoobscura, D. persimilis, D. simulans, D. melanogaster, D. annanassae,* and *D. pallidosa*). In this study, they quantified the strength of postzygotic isolation by investigating hybrid sterility and inviability through mating discrimination experiments, as well as whether postzygotic isolation increases over time. They found that hybrid inviability and sterility was most prominent in the heterogametic sex and that both hybrid sterility and inviability increased over time, which meant that postzygotic isolation gradually increased over time. Subsequent studies on reproductive isolation have based their work on Coyne and Orr (1989) and have extended the quantification and study of reproductive isolation to plants (Ramsey *et al.*, 2003; Moyle *et al.*, 2004; Kay, 2006; Lowry *et al.*, 2008).

Prezygotic barriers (barriers that arise prior to fertilization) include ecogeographic isolation, pollinator fidelity, flowering time, behavioural isolation, and mating system isolation (Dobzhansky, 1935; Coyne and Orr, 2004). Postzygotic barriers (those that act or arise post-fertilization) include intrinsic and extrinsic hybrid inviability. Intrinsic postzygotic barriers are independent of the external environment and result in genetic incompatibilities. Extrinsic postzygotic barriers concern the fitness of the hybrid and its survival in its environment. While prezygotic barriers are generally considered to be more important in preventing gene flow between taxa (Ramsey *et al.*, 2003; Coyne and Orr, 2004; Kay, 2006; Lowry *et al.*, 2008), in some systems it may be that postzygotic isolation is more influential in preventing gene flow. In some studies, postzygotic barriers evolve first when prezygotic barriers are incomplete (e.g., Costa *et al.*, 2007; Yang *et al.*, 2007; Kozak *et al.*, 2012). The importance of either of these reproductive barriers may be different for different lineages. Prezygotic barriers typically evolve at a faster rate than postzygotic barriers (Wiley *et al.*, 2009) and have been the central focus in studying reproductive barriers, because these barriers are easily observed. Postzygotic barriers are mostly genetic in nature (Coyne and Orr,

1989; Coyne and Orr, 2004), and are important for studying hybridization patterns and the genetics of speciation (Presgraves, 2002; Lijtmaer *et al.*, 2003). Due to their primarily genetic nature, postzygotic barriers are often more challenging to study and estimate than prezygotic barriers. Intrinsic and extrinsic postzygotic barriers may vary across different environments and lineages, and their strength and nature may therefore be difficult to generalize across taxa (Lowry *et al.*, 2008). The variation in postzygotic isolation may be due to the distribution in specific alleles that are responsible for intrinsic or extrinsic postzygotic barriers (Sweigart *et al.*, 2007; Barnard-Kubow and Galloway, 2017). In natural populations of *Mimulus nasutus* Greene (Phrymaceae; monkey flowers), and *M. guttatus* DC., for example, the variation in the geographic distribution of incompatibility alleles leads to a variation in postzygotic reproductive isolation (Sweigart *et al.*, 2007). The *hms2* incompatibility allele is widespread and occurs throughout the distribution range of the species, whereas the *hms1* incompatibility allele that interacts epistatically with the *hms2* incompatibility allele, has a more limited distribution within the species. A result of this limit in distribution is that hybrids that occur outside the area of overlap are more fit than hybrids that occur in the area of overlap (Sweigart *et al.*, 2007). In genetic lineages of *Campanulastrum americanum* L. genetic incompatibilities also vary across the distribution range of the species. These differences in genetic incompatibilities are attributed to environmental and demographic differences between the Mountain and Western populations of *C. americanum* (Barnard-Kubow and Galloway, 2017). F_1 hybrids may have incomplete postzygotic isolation and backcross with parental species. This incomplete postzygotic isolation influences subsequent generations (via genetic incompatibilities). Hybrid inviability and sterility may increase in strength over generations, and therefore the overall strength of postzygotic isolation may be underestimated due to an incomplete picture of gene flow (Wiley *et al.*, 2009).

Because of their relative ease to study, prezygotic barriers have generally received more attention than postzygotic barriers, and are often cited as the more important barrier

type, because they are considered to play a larger overall role in reducing gene flow between species (Ramsey *et al.,* 2003; Coyne and Orr, 2004; Kay, 2006; Lowry *et al.,* 2008). For example, after calculating the relative contribution of different barriers to the total reproductive isolation between closely related species *Mimulus lewisii* Pursh and *M. guttatus*, Ramsey *et al.,* (2003) found that cumulatively, prezygotic barriers (e.g., ecogeographic isolation and pollinator fidelity) were stronger than postzygotic barriers. Similarly, Kay (2006) found that habitat differences and floral isolation played an important role in reproductive isolation between closely related *Costus pulverulentus* C. Presl and *C. scaber* Ruiz & Pav. (Costaceae). In both of these studies, despite contributing to the total reproductive isolation, the strength of postzygotic barriers was outweighed by that of prezygotic barriers. In South Africa, prezygotic barriers such as pollinator and phenological specialisation are postulated to be some of the main drivers of plant speciation (Linder, 2003). For example southern African species of the *Disperis* Sw. genus of orchids are characterised by the structure of their lip appendages and by the orientation of the rostellum. These are structures that are related to pollination syndromes and are indicator of the prominent role that pollinator specialisation plays in the speciation of the genus (Manning and Linder, 1992). In the *Argyroderma* N.E.Br. species of the Cape Floristic Region, shifts in flowering times between the different species may result in reproductive isolation (Ellis *et al*., 2007).

Changes in the microspatial composition of habitats may prompt local adaptations in organisms that may lead to habitat isolation (Schluter, 2000; Sobel *et al.*, 2010). For example, *Mimulus lewisii* and a sister species, *M. cardinalis* Douglas ex Benth., are separated by altitude; *M. lewisii* grows between 1600 and 3000 m above sea level (a.s.l.), and *M. cardinalis* grows between sea level and 2000 m a.s.l. (Hiesey *et al.*, 1971). When *M. cardinalis* was transplanted to a higher altitude, it showed poor survival and a later flowering time which had a negative effect on its ability to reproduce at higher altitudes (Hiesey *et al.,*

1971; Ramsey *et al.,* 2003). *Mimulus lewisii* was also found to have a later flowering time when transplanted to a lower altitude (Hiesey *et al.,* 1971; Ramsey *et al.,* 2003). These studies on *Mimulus* species demonstrate that habitat differences such as soil type, microclimate, and available resources may lead to adaptations that could potentially lead to reproductive isolation (e.g., Schluter, 1996). Studying habitat isolation as a reproductive barrier may be challenging for allopatric populations because it is difficult to detect such isolation without doing reciprocal transplants, which are time consuming and costly (Coyne and Orr, 2004). Similarly, in sympatric populations, detecting habitat isolation may be challenging, because the habitat differentiation could be due to competitive differences that are difficult to measure (Schluter, 1996; Glennon *et al.,* 2012).

Phenology relates to the timing of events in an organism's life and affects its ability to reproduce with other taxa. In animals, differences in phenology affects mating times such that breeding times do not overlap, and heterospecific mating cannot occur (Cruzan and Arnold, 1994; Husband and Schemske, 2001; Coyne and Orr, 2004). Phenology governs many aspects such as (not limited to) the unfolding of leaves, flowering emergence, and overall emergence following dormancy, and plays a pivotal role in a plant's biology (Ollerton and Lack, 1992; Elzinga *et al*., 2007). Phenology can also affect flowering time and stigma receptivity to prevent heterospecific pollen transfer (Yost and Kay, 2009). For instance, if flowering times do not overlap, there is no opportunity for gene flow to occur. Ecological differences have led to differences in flowering phenology in sister species *Clarkia xantiana* A. Gray (Onagraceae/gunsight clarkia) and *C. parviflora* Eastw. (Onagraceae) (Runquist *et al.,* 2014). *Costus parviflora* (Costaceae) is adapted to dry conditions, and as a result, develops rapidly and flowers before the onset of water stress (Mazer *et al.,* 2010; Runquist *et al.,* 2014). This rapid development and early flowering in *C. parviflora* prevents the overlap of flowering times with *C. xantiana,* which limits any mating opportunities (Runquist *et al.,*

2014). In the African iris *Gladiolus longicollis* Baker, flowering phenology is an isolating barrier between the two morphotypes found within the species (Anderson *et al*., 2010). Although phenology plays an important role in isolating species, it can be temporally variable, and as such it may not be the most reliable barrier to use in measuring prezygotic isolation (Runquist *et al.,* 2014). Changes in water availability and temperatures over the seasons can have a strong effect on flowering phenology and can lead to earlier and shorter flowering times, which may affect the overall picture of a species' flowering phenology (Mazer *et al*., 2010).

Pollinator isolation presents another important ecological factor in the divergence of plant lineages. Pollinator isolation can manifest through pollination by completely different pollinators that previously visited a particular plant species, different pollination frequencies by the same insects, or through the use of different parts of the same pollinator species by different flowers (Grant, 1993; Schemske and Bradshaw, 1999). Pollinator isolation is also often accompanied by floral divergence to attract specific pollinators. Orchids provide a great example of pollinator isolation because of their strong pollinator specificity (Johnson, 2010; Whitehead and Peakall, 2014). South African carrion mimicking orchids, *Satyrium pumilum* Thunb. mainly attract sarcophagid flies. The orchids offer a medium in which the flies can lay eggs whilst the flies act as pollination agents (van der Niet *et al*., 2011). The pollinator specificity exhibited by these orchids is a strong form of reproductive isolation. In a study of two Australian sexually deceptive orchid species (*Chiloglottis* R.Br. species), Whitehead and Peakall, (2014) found that each species' floral scent attracted a specific wasp pollinator. This specific attraction to floral scent then results in prezygotic isolation. Dell'Olivio *et al.,* (2011) found that following geography, pollinator isolation was the second strongest isolating factor between *Petunia axillaris* (Lam.) Britton, Sterns & Poggenb. and *P. integrifolia* (Hook.) Schinz & Thell. (Solanaceae). These two species occur in similar habitats, but *P. axillaris* is primarily pollinated by hawkmoths and pollen-collecting bees, whilst *P. integrifolia* is

pollinated by a different group of bees (Dell'Olivio *et al.,* 2011). The differences in pollinators account for the low frequency of hybridization in the species' natural environment.

Hybridization occurs when genetically divergent taxa mate to produce offspring (Rieseberg, 1995), and is most likely to occur in recently diverged taxa (Mallet, 2007). Hybridization often occurs between taxa when prezygotic barriers breakdown. These prezygotic barriers often break down when allopatric species come into secondary contact and gene flow occurs between previously isolated species. Estimations of the occurrence of hybridization in plant lineages range from 25% to over 70% (Levin, 2000; Mallet, 2005). The high frequency of the occurrence in evolutionary lineages shows that hybridization has been instrumental in the diversification of species and is acknowledged as a potent evolutionary force (Rieseberg *et al.,* 2003; Mallet, 2005).

Hybridization may have different outcomes in the context of speciation (Abbott *et al.,* 2013). Firstly, in instances where selection and hybridization are at an equilibrium, hybrid zones may form (Barton and Hewitt, 1985; Abbott *et al.,* 2013). These hybrid zones represent a stable state where there is no progression towards speciation or a loss of genetic diversity. One such example of a hybrid zone is formed by three species of Louisiana irises, *Iris fulva* Ker Gawl., *I. hexagona* Walter, and *I.brevicaulis* Raf. where the species occur in sympatry (Arnold, 2006). Alternatively, hybridization may lead to a loss of genetic differentiation through introgression. Taylor *et al.,* (2006) found that the benthic and limnetic species of three-spine sticklebacks (*Gasterosteus aculeatus* L.) in a lake, were collapsing into a hybrid swarm due to increased levels of hybridization. The benthic three-spine stickleback is considered to be a separate species to the limnetic three-spine stickleback due to morphological, ecological, and genetic differentiation (Taylor *et al.,* 2006). An analysis of microsattelites indicated that there was no longer two distinct species of three-spine

stickleback in the lake, but rather a population that had a genetic makeup that was intermediate to that of both three-spine stickleback species. The increased frequency of hybridization is thought to have been induced by the introduction of an exotic fish species into the three-spine stickleback's natural habitat. Further, hybridization may lead to speciation through the development of reproductive barriers between hybrids and their parental forms. One example of hybrid speciation is found in wild sunflowers, where the parental plants *Helianthus annuus* L. and *H. petiolaris* Nutt. (Asteraceae) gave rise to three hybrid species; *H. anomalus* Blake, *H. deserticola* Heiser, and *H. paradoxus* Heiser (Rieseberg, 1991; Rieseberg, 1995). Each of the hybrid species is found in environments different to those of their parental taxa. *H. anomalus* occurs in sand dunes, *H. deserticola* occurs in the desert, and *H. paradoxus* occurs in brackish salt marshes (Rieseberg *et al.*, 2003). In contrast, the parental species *H. annuus* occurs in clay based-soils, whilst *H. petiolaris* occurs in sandy soils (Rieseberg *et al.*, 2003). Hybrid speciation is not only limited to plants but can be seen in other organisms. *Heliconius heurippa* Hewistoni (butterflies in South America) is the hybrid species of *H. melpomene* L. and *H. cydno* Doubleday. *Heliconius heurippa* is reproductively isolated from *H. cydno* by a combination of geography and mate preference. On the other hand, *H. heurippa* and *H. melpomene* are sympatric and are isolated through postzygotic isolation in the form of hybrid sterility (Mavárez *et al.*, 2006).

Hybrid speciation can either be homoploid, where hybridization takes place between individuals of the same ploidy (Rieseberg, 1997). Polyploid hybrid speciation is hypothesised to occur more commonly than homoploid hybrid speciation (Mallet, 2007; Wood *et al*, 2009). Part of the reason for this is that evidence for polyploid hybrid speciation is easy to identify in various genomes. Identifying homoploid hybrids is more difficult in genomes because there may be remnants of historical admixture and confounding factors such as incomplete lineage sorting (Ballard and Rand, 2005; Schumer *et al*., 2014). The role of hybridization in

evolution, and specifically in speciation, is a controversial topic due to the existence of factors such as incomplete lineage sorting. Further, to strengthen support for the importance of hybridization to speciation, traits derived from the hybridization process would have to directly influence or lead to reproductive isolation. Postzygotic reproductive barriers reduce the viability, fitness, or reproductive potential of hybrids (Scopece *et al.*, 2008). Such barriers may be intrinsic and cause hybrids to have inherent developmental deficiencies that cause them to be inviable or sterile (Dobzhansky, 1937; Coyne and Orr, 1998; Orr and Turelli, 2001; Presgraves, 2002; Rogers and Bernatchez, 2006). Alternatively, postzygotic barriers may be extrinsic and lead to ecological and behavioural inviability in hybrids. Hybrids may have traits that are ill-suited to their parental environments and, as a result, have reduced fitness (Dobzhansky, 1937; Coyne and Orr, 1998; Orr and Turelli, 2001; Presgraves, 2002; Rogers and Bernatchez, 2006). Postzygotic isolation has been studied extensively in animals (Coyne and Orr, 1989), and in recent years, more work has been done to try and understand the role of postzygotic isolation in plants (Lowry *et al.*, 2008, Sutherland and Galloway, 2017; Suni and Hopkins, 2018).

Intrinsic postzygotic isolation is independent of the environment (Rogers and Bernatchez, 2006) and refers primarily to hybrid inviability and hybrid sterility. The inviability of hybrids is often based on genetic incompatibilities (Coyne and Orr, 2004; Rogers and Bernatchez, 2006). When alleles that have never interacted before are introduced into a genome through hybridization in secondary contact zones, there is no guarantee that these alleles will be able to function together (Bateson, 1909; Dobzhansky, 1937; Muller, 1940, Coyne and Orr, 2004). Often, alleles that have been introduced into a genome are incompatible with fixed alleles, and have a negative epistatic effect which renders the hybrid partially or completely inviable or sterile (Bateson, 1909; Dobzhansky, 1937; Muller, 1940). These epistatic interactions are known as Dobzhansky-Muller Incompatibilities (DMIs). Extensive studies have been conducted on *Drosophila* to investigate DMIs. For example,

when both pairs of loci responsible for reproduction are homozygous, the hybrids of *D. melanogaster* Meigen and *D. simulans* Sturtevant are inviable, compared to when both pairs of loci are heterozygous, and the hybrids are viable (Orr and Presgraves, 2000). Similarly, in crosses between strains of *Arabidopsis thaliana* L., DMIs activate the immune system of the plant, which leads to necrosis in hybrids. This activation of the immune system is caused by the interaction of two incompatible loci from the nine different strains (Uk–6 through Uk–14; Bomblies *et al.*, 2007).

DMIs are also responsible for asymmetrical reproductive isolation, where interspecific reciprocal crosses have different levels of hybrid inviability or sterility (Turelli and Moyle, 2007). Lowry *et al.* (2008) found that postzygotic barriers are more likely to act asymmetrically than prezygotic barriers. Reproductive barriers are thought to act asymmetrically when the inheritance of genetic factors that control development is unidirectional. This imbalance in the inheritance of genetic factors leads to different levels of hybrid sterility/inviability in reciprocal crosses (Tiffin *et al.*, 2001; Turelli and Moyle, 2007). Wu and Beckenbach (1983) conducted reciprocal crosses between *Drosophila pseudoobscura* Frolova and *D. persimilis* Dobzhansky & Epling, and found asymmetry in male sterility at an X-linked region in one species, but the region did not have the same effect when introgressed into the other species' genome. Asymmetrical postzygotic reproductive isolation occurs similarly in plants. In some instances, the asymmetry may occur only between two sympatric populations of sister species, such as the asymmetry that occurs between *Mimulus guttatus* and *M. nasutus* in the foothills of the Sierra Nevada Mountains (Martin and Willis, 2010). The germination rates of F_1 seeds where *M. guttatus* was the maternal donor germinated at a higher rate than those whose maternal donor was *M. nasutus* (Martin and Willis, 2010).

Extrinsic postzygotic isolation includes barriers such as low fitness of hybrids in an environment, and behavioural sterility (Coyne and Orr, 2004). Hybrids may exhibit low fitness in an environment due to the inability to find a suitable ecological niche (Barton and Hewitt, 1985; Turelli and Orr, 2000). This may occur due to hybrids having an intermediate phenotype to that of their parents, and as such cannot survive in their parental environments (Rundle and Nosil, 2005). For example, in species of sticklebacks, F_1 hybrids that were translocated from the laboratory to the parental environment showed slow growth and development, which suggests that the hybrids have ecological limitations to fitness (Hatfield and Schluter, 1999). Similar results were obtained by Melo *et al.* (2014) between morphologically different populations of *Senecio lautus* Soland. ex Forst. fil (Asteraceae, groundsel) that occupied different habitats. The populations are classified as ecotypes and occur on either sandy dunes (Dune populations), or on rocky headlands (Headland populations). When transplanted to parental habitats, hybrids were selected against due to maladaptation and herbivory.

Postzygotic isolation can also manifest through whole genome duplication (WGD), where an individual has more than two sets of chromosomes. WGD has played a prominent role in the diversification of major organismal lineages (McGrath and Lynch, 2012), including the angiosperms. It is estimated that 70% of plants have polyploidy in their lineage (Stebbins, 1950; Masterson, 1994; Wood *et al.,* 2009). WGD events have also been observed in yeasts (Wolfe and Shields, 1997), amphibians (Morin *et al.,* 2006), and in teleost fishes (Postlethwaite *et al.,* 2000). The presence of WGD events in multiple evolutionary lineages is evidence that polyploidy plays an important role in speciation across the tree of life.

Whole genome duplication represents the most common mode of sympatric speciation in plants (Wendel and Doyle, 2005). Differences in ploidy within populations can affect morphology and floral characteristics. In the South African iris, *Crocosmia aurea* (Pappe ex

Hook.) Planch. tetraploid plants have fewer and larger flowers compared to diploids (Hanneweg *et al*., 2013). Polyploidy can also lead to environmental and geographic expansions (Hijmans *et al*., 2007). The variation in cytotypes in *Oxalis obtusa* Jacq.was found to be correlated with the vegetation type (biome) within the Cape Floral Region (CFR) of South Africa. Hexaploids were most common in the Fynbos biome, whilst tetraploids were the most common cytotype in the Succulent Karoo biome (Krejčíková *et al*., 2013).

Postzygotic isolation between interploid crosses are a result of developmental abnormalities in the endosperm (Ramsey and Schemske, 1998; Laffon-Placette and Köhler, 2016). These endosperm developmental abnormalities are referred to as the 'triploid block', because they prevent the establishment of triploids from interploid crosses between diploids and tetraploids (Ramsey and Schemske, 1998; Ramsey and Schemske, 2002). The developmental abnormalities in the endosperm often result in hybrid seed lethality and seed abortion. A common cause of developmental abnormalities in the endosperm of triploids is parental genomic imbalance. For normal endosperm development to occur, a maternal: paternal genome ratio of 2:1 needs to be present (Ramsey and Schemske, 1998). If this ratio is violated, and the paternal ploidy is higher, endosperm development is more likely to result in the production of inviable seeds (Schatlowski and Köhler, 2012). For example, in *Arabidopsis thaliana*, a parental genomic imbalance changes the expression of the transcription factors, which regulate the cellularization of the endosperm. When the maternal genome is present in excess, transcription factors induce early cellularization. The opposite occurs when there is excess paternal genome (Lu *et al.*, 2012; Schatlowski and Köhler, 2012). The timing of cellularization has an impact on the seed size; a maternal excess is associated with a small seed size, whilst a paternal excess correlates with larger seed sizes (Scott *et al.*, 1998). In some taxa, seeds impacted by parental genomic imbalances may still germinate (Sutherland and Galloway, 2017), but in many cases these imbalances lead to seed abortion, and thereby confer the triploid block in crosses between diploids and tetraploids.

Interestingly, parental genomic imbalances have less severity in crosses between higher ploidy taxa (e.g. 4x–6x) than they do in crosses between lower ploidy taxa (2x–4x) (Sonnleitner *et al.,* 2013; Sutherland and Galloway, 2017). This variation in the impact of imbalances on seed development implies that the level of reproductive isolation in populations with mixed ploidies, may vary, and may even facilitate the formation of higher order ploidies.

In instances where endosperm barriers are overcome, polyploids face the challenge of establishing themselves within progenitor populations. Once triploids have formed and have overcome internal reproductive barriers, they often find themselves in populations in which they are the minority cytotype. The minority cytotype faces a frequency-dependent mating disadvantage which may be a barrier to the establishment of the triploid population (Levin, 1975; Husband, 2000). This exclusion from gene flow decreases the new polyploid's fitness in the progenitor environment. It has been suggested that to try and overcome this obstacle to successful establishment, newly established polyploids may shift from an outcrossing mating system to one where they are self-compatible or reproduce asexually (Ramsey and Schemske, 1998; Otto and Whitton, 2000). In an analysis of 917 species of plants, Robertson *et al.* (2010) found a strong association between polyploidy and self-compatibility in the Solanaceae. Sutherland *et al.* (2018) also found that self-compatibility was strongly correlated with whole genome duplication in *Campanula rotundifolia* L.

Given the difficulty of establishing as a polypoid population (Levin, 1983), it is plausible that newly established polyploids exhibit a shift in mating system from that of the progenitor species. Newly established polyploids often shift from an outcrossing mating system, to one where they are self-compatible. It has been hypothesised that to see a shift from an outcrossing system to a selfing system, a time of at least 40 generations needs to pass for there to be a visible shift in mating system (Cook and Soltis, 1999). Shifting from an

outcrossing mating system to a selfing mating system has been shown to decrease levels of inbreeding in polyploids because self-fertilization increases heterozygosity and results in the purging of deleterious alleles (Barringer and Gerber, 2008). A shift to selfing would decrease the number of hybrid matings between the diploid species and the newly established polyploid, and enhance the reproductive abilities of newly established polyploids (Husband *et al.*, 2008).

1.2 Study taxa

Rhodohypoxis Nel (Hypoxidaceae) is a Drakensberg near endemic genus that comprises six species (Hilliard and Burtt, 1978). The focal species here, *Rhodohypoxis baurii* (Baker) Nel includes three varieties: *Rhodohypoxis baurii* var. *baurii* (Baker) Nel, *R. baurii* var. *platypetala* (Baker) Nel, and *R baurii* var. *confecta* Hillard & B.L Burtt. A second study species, *Rhodohypoxis milloides* (Baker) Nel, is related to *Rhodohypoxis baurii* (Uys and Cron, unpublished data). *Rhodohypoxis* plants are short, geophytic herbs with contractile roots. The leaves are basal and are either erect or spread on the soil surface. The indumentum on the leaves and pedicel varies from dense to sparse according to variety and may also be present on the abaxial surface. Flowers may be white, pink or a deep magenta.

R. baurii var. *baurii* is found in moist, shaded areas at the base of cliff faces and has magenta flowers (Figure 1a; Hilliard and Burtt, 1978). *R. baurii* var. *baurii* is predominant in the southern Drakensberg, and rarely extends into the northern Drakensberg. *R. baurii* var. *baurii* can be found at altitudes between 1350 and 2500 m a.sl. Its leaves are more erect and thinner than those of *R. baurii* var. *platypetala.* Both of these varieties have dense trichomes on the abaxial leaf surface, and sparse trichomes on the adaxial leaf surface.

R. baurii var. *platypetala* is the most widespread variety, and ranges from the southern Drakensberg to parts of the northern Drakensberg at altitudes between 900 m above sea level (a.s.l.) and 2250 m a.s.l (Hilliard and Burtt, 1978). The flowers of *R. baurii* var.

platypetala are predominantly white (Figure 1b) to sometimes very pale pink. This variety can often be found in dry, rocky soils. The leaves in *R. baurii* var. *platypetala* are a dull grey-green colour, broad, and often spread on or near the soil surface.

R. baurii var. *confecta* has the most restricted range of the three variants, and occurs only in eight known locations in the northern Drakensberg (and into Swaziland) at altitudes above 2200 m a.s.l. The flower colour of *R. baurii* var. *confecta* ranges from white to bright pink (Figure 1c). This variety can be found in habitats that are intermediate to those of *R. baurii* var. *baurii* and *R. baurii* var. *platypetala*. *R. baurii* var. *confecta* has hairy, semi-erect leaves. *R. milloides* has magenta (Figure 1d) to rarely deep red flowers. It is found in marshy areas and has thin, bright green, erect, glabrous leaves. *R. milloides* is found at altitudes between 1350 and 2500 m a.s.l.

The flowers of *R. milloides* and the *R. baurii* varieties are hermaphroditic, typically with six anthers arranged in two rows of three, and are situated in close proximity to the stigma. The stigma is attached to a short style (~2mm), and is split into two lobes in the three *R. baurii* varieties, or three lobes in *R. milloides.* The arrangement of the reproductive structure and flower colour is important for deciphering potential mating systems, especially since there are no known pollinators of *R. baurii* (Pers. obs.). Plant reproductive structures promote mating, and the diversity in the form and function of floral displays is associated with the diversity in mating strategies (Barret, 2010).

Figure 1. a) *Rhodohypoxis baurii* var. *baurii*. b) *Rhodohypoxis baurii* var. *platypetala.* c) *Rhodohypoxis baurii* var. *confecta*. d) *Rhodohypoxis milloides*

Previous taxonomic work on *Rhodohypoxis* highlighted that several species naturally hybridize and that introgression likely occurs (Hilliard and Burtt, 1978, Uys and Cron, unpublished data). Putative hybrids between the three *R. baurii* varieties and between *R. baurii* var. *platypetala* and *R. milloides* have been observed in the field (Hilliard and Burtt, 1978; Uys and Cron, unpublished data). These putative hybrids were detected in a separate, intermediate environment and exhibit physical characteristics that are intermediate between the parent species. The occurrence of these hybrids has led to questions regarding the taxonomy of the genus, as well as the presence or absence of reproductive barriers between these varieties and species. The existence of hybrids in natural populations strongly suggests that divergence is recent within the genus, and that reproductive barriers are still forming (Rieseberg *et al.*, 1993; Yi and Maki, 2015). On several recent visits to sites where hybrids were previously observed no hybrids could be found (K. Glennon, pers. comm.). This led to questions about the persistence of the hybrids within *Rhodohypoxis* populations and suggests that postzygotic barriers may inhibit the long term survival of such hybrids.

1.3. Aim and objectives

The main aim of this study was to test for the presence of postzygotic reproductive isolation among *Rhodohypoxis baurii* varieties and between the varieties and *R milloides*, with three distinct objectives. The first of these objectives was to examine postzygotic reproductive barriers through controlled crossing experiments in the greenhouse. Crossing experiments provide the opportunity to study and directly observe hybrids and postzygotic isolation in a controlled, laboratory environment. Results from these crossing experiments allowed us to assess the viability of *R. baurii* hybrids. The second objective was to characterize gene flow among *R. baurii* varieties and *R. milloides*. To achieve this end, patterns of gene flow were characterized using amplified fragment length polymorphisms as genetic markers. Characterizing gene flow among populations is a first step to identifying patterns of diversification. These patterns then enabled us to infer past gene flow patterns, which revealed the presence of reproductive barriers between the four taxa. The third objective was to estimate ploidy levels for each *R. baurii* variety and *R. milloides* using flow cytometry. Ploidy may play a role in reproductive isolation when the taxa being crossed have different ploidy levels. Estimating ploidy levels will help infer whether ploidy plays a role in reproductive isolation between the three *R. baurii* varieties and *R. milloides.*

CHAPTER 2: MATERIALS AND METHODS

2.1. Crossing study

Plant collection

Twenty individuals of *Rhodohypoxis baurii* var. *baurii* and 20 individuals of *Rhodohypoxis baurii* var. *platypetla* were collected from Hebron Farm near Kokstad, KwaZulu-Natal in 2015 and 2016. A further 20 individuals of *Rhodohypoxis baurii* var. *platypetala* and 20 individuals of *Rhodohypoxis milloides* were collected from the same site, Sunset Farm, near Bulwer, KwaZulu-Natal, during the same two years. A further 20 individuals of *Rhodohypoxis baurii* var. *confecta* were collected from Sentinel Peak in the Drakensberg in 2017. The individuals were potted in the greenhouse and maintained in the outdoor garden at the University of the Witwatersrand, Johannesburg before being moved into the rooftop greenhouse at Wits. The plants were allowed to acclimatize for one year under controlled watering conditions before they were incorporated into the crossing study. Plants received ambient light conditions, the greenhouse was temperature controlled (though temperature ranged from 18–37°C during the flowering season), and plants were watered with approximately 200ml water twice per day.

Experimental design to test reproductive success between lineages

Crosses were done within varieties, between varieties, and between species (Figure 2). In addition, five to eight individuals of each taxon were selfed (pollen from same plant deposited onto the stigma of the same flower) to test whether plants were self-compatible.

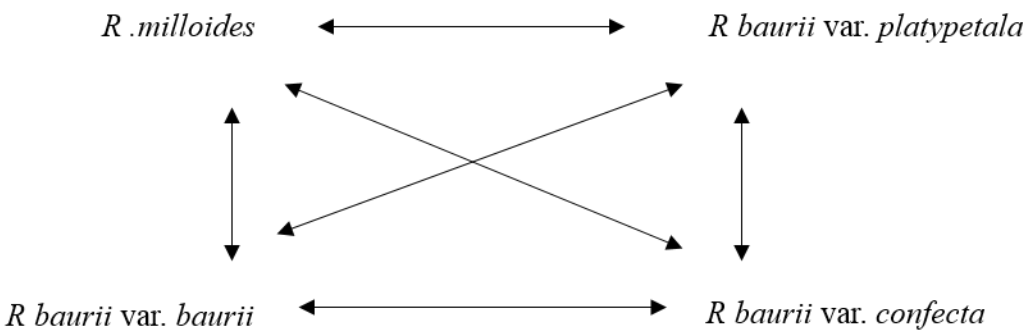

Figure 2. A summary of all the crosses made between different *Rhodohypoxis* taxa, with arrows indicating that all crosses were reciprocal.

Before crosses were conducted, flowers were covered in small cages made from plastic cups with windows cut into the sides and the top to allow sunlight to enter and to prevent overheating (Figure 3a). These windows were covered with a fine mesh to exclude pollinators, but this mesh did not prevent watering from the irrigation system. Individual plants were randomly chosen for crosses based on availability of flowers that had been open for at least 2 days. Flowers were emasculated using fine forceps (BioQuip #4535 Swiss), which were cleaned with 70% ethanol between crosses to prevent pollen contamination. The emasculated flowers were then used as maternal plants, while pollen from the emasculated plant was used as the paternal donor for other maternal plants. A fine paintbrush (Dala Gold 756 round brush, size 4) was used to deposit pollen onto stigmas. Paintbrushes were also cleaned between crosses using 70% ethanol to prevent pollen contamination. Once pollinated, the flowers were marked with coloured rubber bands to separate them from non-pollinated flowers, and placed back under the cages. To test for autogamous selfing, a single flower was emasculated, and had its own pollen deposited onto its stigma. Pollen deposition occurred when the pollen was "fluffy", approximately one day after the opening of the flower (there was an observable 'slit' in the anther to release pollen, pers. obs.). Stigmas were also presumed to be receptive one day after the opening of the flower. All pollinated flowers were then placed under a larger mesh cage to ensure that pollinators were excluded.

Potentially successful crosses were identified by having swollen receptacles. Once flowers scenesced, seeds were collected by removing the dried out flower from the pedicel to reveal the capsule three weeks after pollination (Figure 3b). Seeds did not often dislodge themselves from capsules, so manual manipulation was required to remove seeds. After collection, seeds were counted to compare the average seed set between flowers. To quantify hybrid inviability, 10% of the seeds from each cross were sowed at depth of 5 mm in herb potting mixture (Healthy Living Herbs™) in individual pots. The seeds were watered twice a day in the greenhouse for five minutes in the morning (approximately 09:30) and again in the afternoon (approximately 15:30) by an automated watering system (approximately 200ml each irrigation). Germination success was recorded as the emergence above ground after four weeks.

Figure 3. a & b) Plastic cages used to cover *Rhodohypoxis* flowers after crossing experiments had been done. c) A flower that has detached from the stem to reveal a capsule full of seeds.

2.2. Pollen viability

Pollen viability was tested with analine blue stain. Anthers from a hybrid between *R. milloides* x *R. baurii* var. *platypetala* (SM12 x PH3), and anthers from *R. baurii* var. *baurii* were separately placed in ~100 µl of aniline blue and left overnight. Tubes that stored pollen grains were vortexed to enable even distribution of pollen throughout the stain solution and 40µl were aliquoted onto a microscope slide for observation using a compound light

microscope (Zeiss Axio Imager 2) at 400x magnification. Pollen was considered viable if pollen grains were stained and nonviable pollen appeared to be transparent. The total number of stained and unstained pollen grains were counted for each sample in one field of view to estimate percent viability.

2.3. Flowering Phenology

Phenological data of flowers were collected in the greenhouse through daily observations of plant growth during the flowering season (late August – early February). The date of the first flower opening was recorded for each taxon and the total number of flowers per plant per variety over one flowering period (2018) was calculated.

2.4. Characterising gene flow

Assessing gene flow among populations can help to elucidate past gene flow patterns that have subsequently shaped the trajectory of species. Patterns of introgression and hybridization can be elucidated using molecular techniques such as the use of amplified fragment length polymorphisms (AFLPs). AFLPs are based on the detection of homologous fragments in the genome that are amplified using the polymerase chain reaction (PCR) (Vos *et al.,* 1995). These regions in the genome are then restricted using restriction enzymes one restriction enzyme with an average cutting frequency (e.g., MseI), and one restriction enzyme with a high cutting frequency (e.g., EcoRI) (Savelkoul *et al.,* 1999). These fragments are then assessed for the presence or absence in the sampled individuals.

AFLP is a fingerprinting technique that has been widely used to assess genetic diversity, characterize population structure, and elucidate phylogenetic relationships (Mueller and Wolfenbarger, 1999; Bensch and Akesson, 2007; Meudt and Clarke, 2007). This wide use of AFLP can be attributed to the reproducibility and robustness of the techniques due to the use of stringent PCR annealing temperatures (Blears *et al.,* 1998). An additional advantage of the AFLP approach is its capacity to reveal a high number of polymorphisms with small quantities of DNA (0.05-0.5µg) at a relatively low cost (Blears *et al.,* 1998;

Blignaut *et al.,* 2013). Unlike other molecular methods, AFLPs do not require prior knowledge regarding the targeted genome (Vos *et al.,* 1995).

In a comparison of three DNA based techniques (Random Amplification of Polymorphic DNA (RAPD), Inter Simple Sequence Repeats (ISSR), and Amplified Fragment Length Polymorphism (AFLP)) to characterise genetic variation in *Dactylis glomerata* L. (cat grass), Costa *et al.* (2016) found that AFLPs were most efficient at detecting polymorphisms. RAPDs produced 97 bands of which 40 were polymorphic, compared to AFLPs, which produced 100 bands of which 92 were polymorphic (Costa *et al.,* 2016). AFLPs have also been used to decipher the genetic structuring of species within the *Draba* L. (Brassicaceae) genus. The genus consists of cryptic species. Through the use of AFLP markers, the authors were able to decipher phylogeographic structures and infer past patterns of dispersal and gene flow (Skrede *et al.,* 2009). Both of these studies demonstrate that the AFLP technique has the ability to produce informative results without compromising the robustness of the technique.

DNA extractions

DNA was extracted using the CTAB (cetyl trimethylammonium bromide) method adapted from Doyle and Doyle (1987). The CTAB mixture facilitates the breakage of polysaccharides from the plant cell wall, while the chloroform-Iso Amyl separates the cell DNA from the rest of the cellular debris. Alcohol 2-Isopropanol is used to precipitate out the DNA, and ethanol is used to clean the DNA. Genomic DNA samples were quantified using a micro-volume UV-Vis spectrophotometer (Nanodrop, Thermo Fisher Scientific). DNA was classified as being of "good quality" if the A260/280 ratio was above 1.6 and had a DNA concentration of at least 100ng/µL.

Genotyping parental plants

The AFLP protocol used in this study followed Blignaut *et al.,* (2013), and comprises four steps: 1) restriction digest, 2) ligation, 3) pre-amplification, and 4) selective amplification. The restriction enzymes, EcoRI and MseI were used to digest 100ng/µl of genomic DNA. EcoRI and MseI recognises the palindromic sequences, 5'-GAATTC-3', 5'-TTAA-3', respectively. The EcoRI digestion reaction mixture consisted of 0.25 µL of 5 U of EcoRI, 2 µL of 1X EcoRI buffer, 16.75 µL of distilled water, and 1 µl of at least 100 ng/µl of DNA to make up a total volume of 20 µL per reaction. The mixture was then left to digest at 37°C in a water bath for 1 hour. After incubation, 1 µL of 5 units of MSe1, 3 µL of 1X Cutsmart buffer, and 6 µL of distilled water was added directly to the EcoR1 mixture. The mixture was then incubated at 37°C for 1 hour whereafter it was 65°C for thirty minutes.

After digestion, adaptors that are complementary to either MseI or EcoRI were ligated to the digested DNA fragments. For the ligation mixture, 1U of T4 Ligase, 1X T4 DNA Ligase Buffer, 5 µM MseI adaptor, 5 µM EcoRI adaptor, and 6.9µL distilled water were added to the digestion mixture. The digestion-ligation mixture was then left to incubate overnight at 4°C. After incubation, 5 µL of the digestion-ligation mixture was run on a 1% agarose gel to check for the presence of smears between 100 and 500 bp. These smears indicated the presence of DNA fragments.

For pre-selective PCR amplification, an undiluted sample of the digestion-ligation mixture was used as a template. The pre-selective PCR master mixture contained 1X Amplicon Taq Master Mix Red, 5.5 µL distilled water, 1 µM MSeI+0 primer, 1 µM EcoRI+0 primer, and 2.5 µL of the diluted digestion-ligation reaction mixture. Pre-selective PCR amplification was conducted following Blignaut *et al* (2013) with an initial denaturing step at 94°C for 5 minutes, followed by denaturation at 94°C for 30 seconds, annealing for 30 seconds at 56°C, elongation at 72°C for 30 seconds, repeated for 23 cycles. The final

elongation step was set at 60°C for 30 minutes. After pre-selective amplification, 5 µL of each PCR product was run on a 1% agarose gel at 45V for one hour. After running the gel, the gel was checked for the presence or absence of smears between 100 bp and 500 bp to indicate a successful pre-selective amplification.

Following pre-selective amplification, undiluted PCR products were used as templates in the selective amplification. Each 20µL selective PCR reaction consisted of 2X Amplicon Taq Polymerase Master Mix, 10µM fluorescently labelled EcoRI+ NNN (see Table 1 for a list of primers), 1 µL of 10 µM unlabeled MSeI-+CTT, and 5 µL of diluted pre-selective PCR product. The selective PCR cycle was run similarly to the pre-selective PCR cycle, but with 30 cycles instead of 23 cycles. Successful selective PCR products were sent for fragment analysis at the Central Analytics Facility at the University of Stellenbosch, South Africa.

Table 1. Primers from Blignaut et al. (2013) that were used in selective amplification of the PCR product of *R. baurii* varieties and *R. milloides.*

Primer name	Sequence (5'-3')
MseI+CTT	GATGAGTCCTGAGTAACTT
EcoRI-ATG	GACTGCGTACCAATTCATG
EcoRI-CAT	GACTGCGTACCAATTCCAT
EcoRI-AAT	GACTGCGTACCAATTCAAT

2.5. Estimation of ploidy

Estimating ploidy is important in studies pertaining to reproductive isolation because differences in ploidy can contribute to decreasing gene flow between taxa (Ramsey and Schemske, 1998; Hersch-Green, 2012). In this case, fresh leaf tissue samples from each *Rhodohypoxis baurii* variety and *R. milloides* were sent to the Royal Botanic Gardens, Kew in London for the estimation of ploidy using a Partec Flow Cytometer (Partec Inc., Germany). Flow cytometry is a method that uses a combination of staining and fluorescence to determine genome size and ploidy (Gregory, 2004). Nuclei were isolated from the cell by cutting up leaf tissue and suspending it in Otto's buffer that releases the nuclear content

(Otto, 1992; Dolezel *et al.,* 1998). The nuclear content was then stained with a fluorescent dye (Propidium iodide) that binds to the DNA. The flow cytometer passes the nucleic content through a sequence of lights, mirrors and amplifiers that converts the emitted fluorescent light into a digital signal that is measured against a known genome size in order to conclude the genome size of (in this case, *Pisum sativa* L.) the species of interest (Dolezel *et al.,* 1998).

2.6. Data analysis

Seed set and germination

Differences in seed set and germination between cross types were analysed using a generalised linear model (GLM) in R (R Core Team, 2016) using the lme4 package (Bates *et al.,* 2015) following Suni and Hopins (2018). This approach helped account for the unbalanced seed set results from different crosses. To evaluate differences in seed set between intervarietal, intravarietal, and interspecific crosses, I ran four GLMs (one for each taxon) using the lme4 package (Bates *et al.,* 2015) in R (R Core Team, 2016). In each model, I set the number of seeds as the dependent (response) variable and the paternal taxon as the independent (predictor) variable. The number of crosses per individual were included as an offset, and the maternal plant identity was included as a random effect. I then used the lm() function in R to test for significant differences between intervarietal, intravarietal, and interspecific crosses.

Molecular data analysis

Chromatographs were scored for the absence (zero) or presence (one) of fragments for each primer pair using the GeneMarker software (SoftGenetics, State College, PA). Fragments were scored as present when the relative fluorescence unit (RFU) level was at least 100. If peaks were found at consecutive base pairs, only the peak with the highest RFU was scored, and the other peaks were interpreted as stutter. The final result after scoring was a binary matrix which was formatted for analysis in relevant software.

Population differentiation

Measures of population differentiation (F_{ST}), heterozygosity (H_0), and genetic distance (D) were used to describe population structure and differentiation and were calculated using GenAlEx v. 6.5 (Peakall and Smouse, 2012).

To characterise population structure, I used STRUCTURE version 2.3.4 (Pritchard *et al.*, 2000, Falush *et al.*, 2003a) which uses multilocus genotype data to cluster individuals into populations using a Markov chain Monte Carlo algorithm. The algorithm estimates the probability of each individual of belonging to each genetic cluster (Pritchard *et al.*, 2000; Falush *et al.*, 2003b). The estimation of the probability of belonging to a cluster for each individual is done on the basis of *K*, which is the number of assumed populations. Some applications of STRUCTURE include identifying cryptic population structure, detecting migrants in populations, and inferring historical admixture (Falush *et al.*, 2007). For example, through the use of STRUCTURE, populations of Indian Himalayan *Arabidopsis thaliana* were found to have genetic variation that is structured by altitude and geography (Tyagi *et al.*, 2016). STRUCTURE has also been used to characterise the genetic structure of human populations (Rosenberg *et al.*, 2002). The use of STRUCTURE in various studies shows the program's ability to provide robust, informative results that can be used to make reliable conclusions about the structure of populations.

When running the STRUCTURE analysis, a haploid dataset was used, and -9 was set as the value for missing data. A haploid dataset was used because dominant markers such as AFLPs do not distinguish between heterozygotes and homozygotes and present only a presence or absence of each fragment for each individual. Each row from the dataset was recognised as a single individual by the program. Five values of K (K = 1-5) were explored because the *R. baurii* varieties come from two separate localities (Sunset Farm and Hebron Farm), whilst *R. milloides* is a different species from the varieties and is expected to have a

different population structure. An analysis with a burnin period of 65000 and 650000 MCMC (Markov chain, Monte Carlo) repetitions, with 4 iterations was run on each cluster. The STRUCTURE model that was used assumes that allele frequencies are correlated among populations due to shared ancestry. The end result is a clustering of individuals according to the degree of similarity of their genotypes.

CHAPTER 3: RESULTS

3.1. Self and cross-compatibility experiments

Seed set

In the experiment to test for compatibility within a taxon and between varieties and/or species of *Rhodohypoxis*, crosses between *R. milloides* and *R. baurii* var. *platypetala* produced the highest number of seeds (n = 179, Table 2). The second highest number of seeds was produced by crosses between *R. baurii* var. *platypetala* and *R. baurii* var. *baurii* (n = 167, Table 2). A notable trend is that all crosses involving *R. baurii* var. *platypetala* as either the maternal or paternal plant, had a relatively high seed set compared to crosses involving *R. baurii* var. *baurii* and *R. baurii* var. *confecta*. Collectively, flowers that were autogamously selfed produced more seeds across taxa than either intravarietal or intraspecific crosses. On average, intervarietal crosses (mean= 138 ± 90, Figure 4) produced the most seeds, followed by interspecific crosses (mean = 123.5 ± 16.5, Figure 4). Autagomously selfed crosses produced more seeds (mean = 106 ± 61) than intraspecific crosses (crosses between *R. milloides* individuals) (mean = 34.9 ± 9.5, Figure 4).

Table 2. The number of seeds produced by crosses between *Rhodohypoxis* varieties and *R. milloides*. The values in bold indicate the number of seeds that were produced in each cross type, alongside the number of crosses between taxa.

	Paternal taxon			
	R. baurii var. *baurii*	*R. baurii* var. *platypetala*	*R. baurii* var. *confecta*	*R. milloides*
Maternal taxon				
R. baurii var. *baurii*	2, **5**	12, **90**	2, **35**	1, **20**
R. baurii var. *platypetala*	12, **167**	4, **113**	1, **30**	8, **57**
R. baurii var. *confecta*	0, **0**	1, **3**	0, **0**	4, **29**
R. milloides	0, **0**	18, **179**	1, **8**	5, **19**

Table 3. Results from a generalized linear model that tested the effect of maternal taxon on seed set in heterospecific crosses between *Rhodohypoxis* varieties and *R. milloides.* Significant probability values are marked with an asterisk. The maternal plant is listed first and the paternal plant is listed second (maternal<paternal).

Cross type	z	SE	t	P-value	N seeds produced
baurii<confecta	13.65	4.03	3.90	0.20*	46
baurii<milloides	11.50	4.03	2.85	0.40*	20
baurii<platypetala	2.00	4.03	0.66	0.54	120
platypetala<baurii	8.61	8.57	1.01	0.34	229
platypetala<confecta	21.61	17.53	1.23	0.25	30
platypetala<milloides	4.89	8.90	0.55	0.60	93
milloides<confecta	-21.00	13.71	-1.53	0.22	8

There was an indication of slight unidirectional seed production when controlling for maternal taxon identity in crosses between *R. baurii* var. *baurii* and *R. baurii* var. *confecta* (z = 13.65, df = 5, SE = 4.03, P = 0.20, Table 3), as well as in crosses between *R. baurii* var. *baurii* (z = 11.50, df = 10, SE = 4.03, P = 0.40, Table 3) and *R. milloides*. In addition, I observed that in one reciprocal cross between *R. baurii* var. *platypetala* (Karkloof 10) and an F_1 hybrid of *R. milloides* x *R. baurii* var. *platypetala*, seeds were only produced in the cross where *R. baurii* var. *platypetala* was the maternal plant. In this single cross, six seeds were produced, with two of the six seeds appeared to have been aborted. None of the other cross combinations showed any significant asymmetry. When fully developed, *Rhodohypoxis* seeds are mamillate (with nipple-like protuberances), a black testa, and measure approximately 2 mm in diameter. The aborted seeds appeared white and were less than 2 mm in diameter. No seeds were produced in the single reciprocal cross where the F_1 hybrid was the maternal plant.

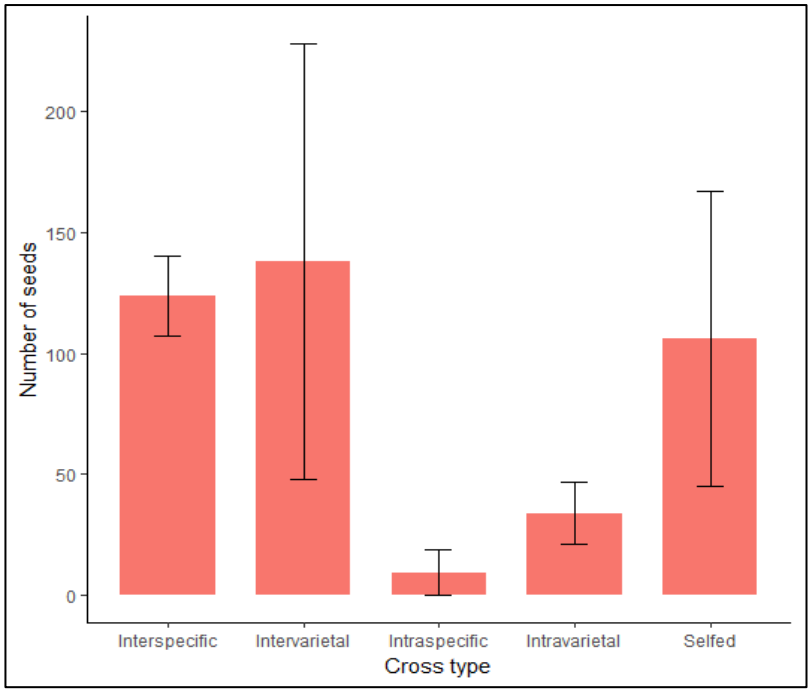

Figure 4. The average number of seeds produced by each cross type. Interspecific crosses (n = 8) are between two *R. baurii* varieties (*R. baurii* var. *platypetala* and *R. baurii* var. *confecta*) and *R. milloides*. Intervarietal crosses (n = 13) are between all three *R. baurii* varieties. Intraspecific crosses (n = 2) are between individuals of *R. milloides.* Intravarietal (n = 4) crosses are between individuals of the same variety, and each selfed cross (n = 5) was autogomously hand-pollinated. The error bars represent the SE for each cross type.

3.2 Flowering phenology

The flowering times of *R. baurii* and *R. milloides* overlapped during the 2018 flowering season. *R. baurii* var. *baurii* flowers first in late August, followed by *R. baurii* var. *platypetala* in the first week of September. *R. baurii* var. *confecta* flowers late in September, whilst *R. milloides* starts flowering in the last week of October.

3.3. Seed viability

To test for seed viability, 10 % of the total seeds collected per cross were germinated, and the percentage germination for each cross type was recorded. In total, 47.25% of seeds from all cross types germinated (Table 4), with interspecific crosses having the highest germination rate (68.86%, Table 4), followed by intraspecific crosses with 42.86% of seeds (Table 4). Intravarietal crosses had a percentage germination of 39.13% (Table 4). Intervarietal crosses had the lowest percentage germination (38.14%, Table 4).

Two of the germinated hybrid seedlings flowered, one in 2018 (PH3xSM12) and one hybrid in 2019 (SM12xPH3). Both seeds were the result of reciprocal crosses between the same individuals: *R. milloides* from Sunset Farm and *R. baurii* var. *platypetala* from Hebron Farm. Both hybrid flowers were a light pink colour, which is intermediate to that of the parent plants.

Figure 8. Example of two hybrids that resulted from a reciprocal cross between parents. a) A hybrid of *R. milloides* from Sunset Farm and *R. baurii* var. *platypetala* from Hebron Farm, with *R. baurii* var. *platypetala* as the maternal plant (PH3xSM12. b) A hybrid of an *R. baurii* var. *platypetala* from Hebron Farm with *R. milloides* from Sunset Farm as the maternal plant (SM12xPH3).

Table 4. The percent germination in different cross types of *Rhodohypoxis baurii.* Interspecific crosses are between two *R. baurii* varieties (*R. baurii* var. *platypetala* and *R. baurii* var. *confecta*) and *R. milloides*. Intervarietal crosses are between *R. baurii* varieties. Intraspecific crosses are between individuals of *R. milloides.* Intravarietal crosses are between individuals of the same variety.

Cross type	% germination	n seeds
Interspecific	68.86	83
Inter-varietal	38.14	118
Intraspecific	42.86	14
Intra-varietal	39.13	23

3.4. Pollen viability

Pollen obtained from a hybrid (PH3xSM12) of *R. milloides* and *R. baurii* var. *platypetala* appeared less viable than pollen obtained from an intravarietal cross between individuals of *R. baurii* var. *baurii*. The hybrid pollen contained 128 grains of pollen in one field of view, whilst *R. baurii* var. *baurii* had 370 grains of pollen in one field of view. Of the 128 pollen grains counted for the hybrid, 43.75% of the grains were viable. Of the 370 pollen grains counted, 94.87% of the pollen grains were viable. The hybrid pollen also had fewer stained pollen than *R. baurii* var. *baurii* pollen, which suggests lower viability of the hybrid pollen (Figure 5a & b; Kearns and Inouye, 1993).

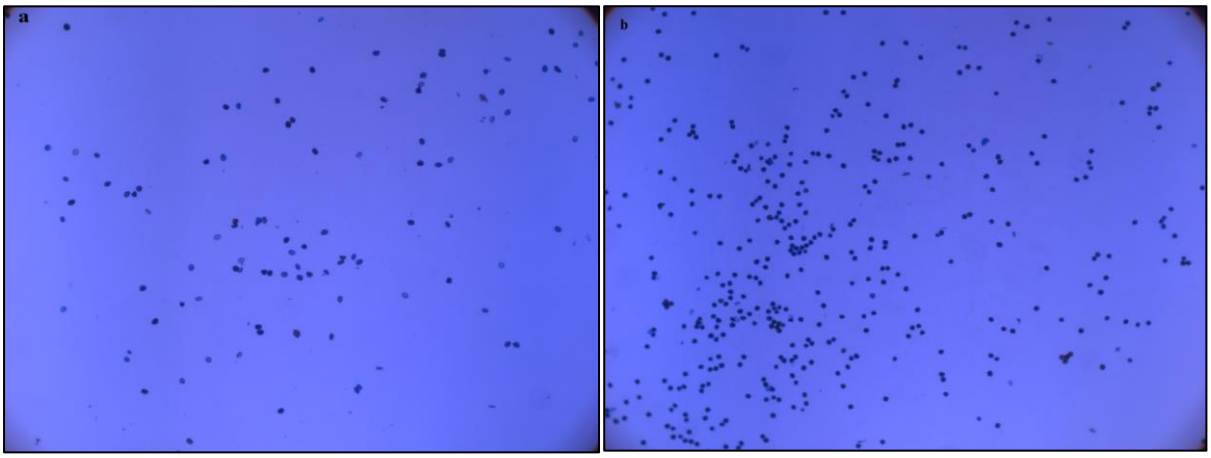

Figure 5. A comparison of pollen formed by (a, left) an interspecific hybrid (*R. milloides* x *R. baurii* var. *platypetala*), and (b, right) *R. baurii* var. *baurii*. There are fewer interspecific hybrid pollen grains compared to *R. baurii* var. *baurii* pollen grains. Fewer interspecific hybrid pollen stained relative to *R. baurii* var. *baurii* pollen.

3.5. Characterising gene flow

Population variation

Genetic distance was greatest between *R. baurii* var. *platypetala* from Sunset Farm and *R. milloides* from Sunset Farm (0.061, Table 4). *R. baurii* var. *platypetala* from Hebron was more genetically similar to *R. milloides* from Sunset Farm (0.007, Table 4) than to *R. baurii* var. *platypetala* from Sunset Farm (0.000, Table 4), despite the difference in location.

94.12% of loci across all taxa in the Hebron Farm population were found to be polymorphic, while 100% of the loci across both *R. milloides* and *R. baurii* var. *platypetala* in the Sunset Farm population were found to be polymorphic. The mean percentage of polymorphic loci was 97.06% across all taxa in both the Sunset Farm and Hebron Farm populations.

Table 5. Unbiased pairwise genetic distance between varieties of *R. baurii* and *R. milloides* from two different populations, Hebron Farm (H), and Sunset Farm (S) in KwaZulu-Natal, South Africa.

	R. milloides (S)	*R. baurii* var. *platypetala* (S)	*R. baurii* var. *platypetala* (H)	*R. baurii* var. b*aurii* (H)
R. baurii var. *baurii* (H)				0
R. baurii var. *platypetala* (H)			0	0
R. baurii var. *platypetala* (S)		0	0	0
R. milloides (S)	0	0.065	0.007	0.061

Table 6. Mean genetic variation over loci per taxon at Hebron Farm and Sunset Farm. N_a = number of alleles, N_e = number of effective alleles, I = Information index, h = gene diversity, uh = unbiased gene diversity.

Taxon	N_a	N_e	I	h	uh
R. baurii var. *baurii* (Hebron Farm)	1.725	1.530	0.444	0.302	0.369
R. baurii var. *platypetala* (Hebron Farm)	1.922	1.633	0.532	0.362	0.414
R. baurii var. *platypetala* (Sunset Farm)	1.725	1.448	0.402	0.269	0.337
R. milloides (Sunset Farm)	1.882	1.675	0.559	0.383	0.447

R. baurii var. *baurii* from Hebron Farm and *R. baurii* var. *platypetala* from Sunset Farm showed low mean genetic diversity (uh = 0.369, uh = 0.337, Table 6). The highest genetic diversity was detected in the *R. milloides* population from Sunset Farm (uh = 0.447, Table 6), while *R. baurii* var. *platypetala* from Hebron Farm had the second highest diversity (uh = 0.414, Table 6). The % polymorphic loci was highest in *R. milloides* (94.12%) from Sunset Farm, followed by R. *baurii* var. *platypetala* from Hebron Farm (92.16%). *R. baurii*

var. *baurii* from Hebron Farm has 76.47% polymorphic loci. The taxon with the lowest % polymorphic loci is *R. baurii* var. *platypetala* from Sunset Farm (72.55%).

Population structure

The STRUCTURE analysis revealed two genetic clusters out of the possible five clusters evaluated ($\Delta K = 2$; Figure 6). The Sunset Farm population shows less admixture than the Hebron Farm population. The majority of admixture occurred between *R. baurii* var. *baurii* and *R. baurii* var. *platypetala* individuals from Hebron Farm. Of the 15 individuals from the Sunset Farm population (eight *R. milloides* and seven *R. baurii* var. *platypetala*), only three *R. baurii* var. *platypetala* individuals show no admixture. All *R. milloides* individuals show some level of admixture with *R. baurii* var. *platypetala* from Sunset Farm. Four of the seven individuals of *R. baurii* var. *platypetala* from Sunset Farm share a majority of their genotypes with *R. milloides* individuals (Figure 7a).

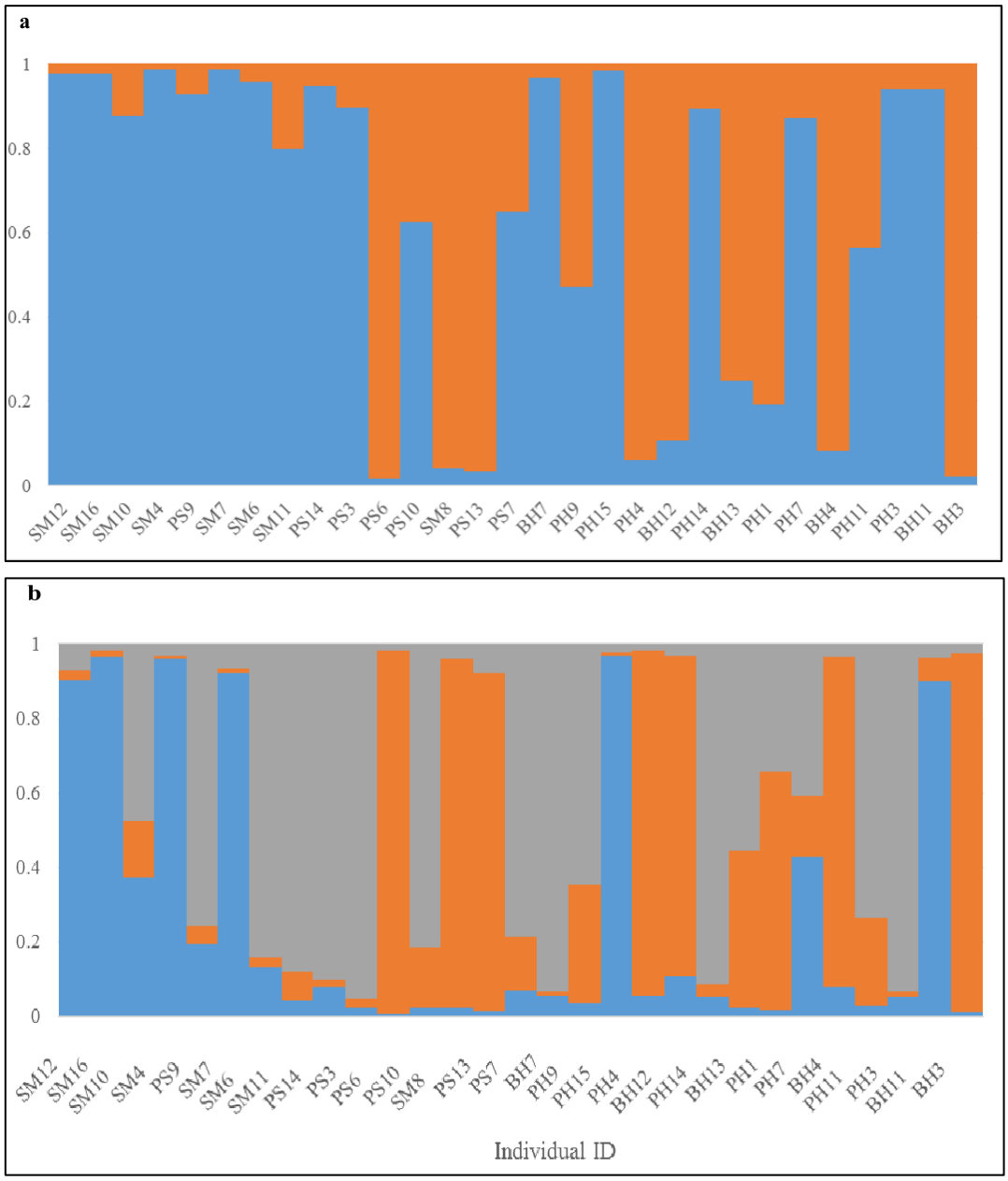

Figure 6. a) A barplot for two genetic clusters identified by Structure Harvester (deltaK = 2) shows admixture among 29 individuals.. Cluster 1(in blue) consisted of individuals of *R. milloides* (SM) and *R. baurii var*. *platypetala* (PS) collected from Sunset Farm. Cluster 2 (in orange) consisted of individuals of *R. baurii* var. *baur*i (BH) and *R. baurii* var. *platypetala* (PH) collected from Hebron farm. Each bar represents an individual plant. b) Population structure when K=3. The x-axis shows each individual within either of the genetic clusters.

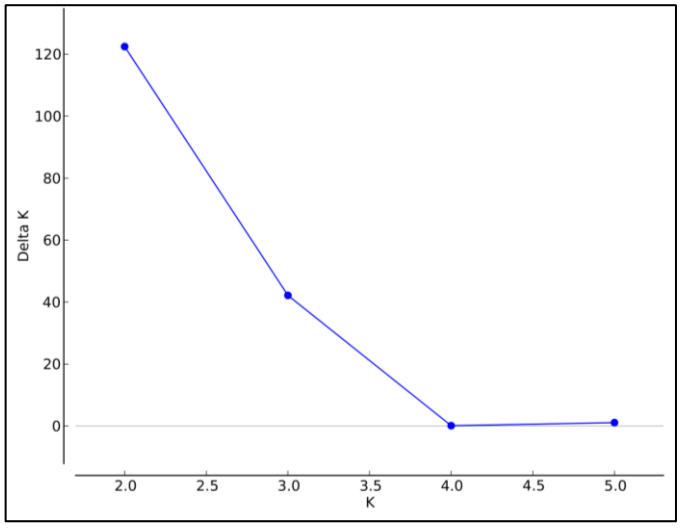

Figure 7. Five possible genetic clusters were assessed using Structure and Structure Harvester. A Delta K value of two indicates that there are two genetic clusters represented in the dataset.

3.6. Ploidy

Ploidy levels varied among *R. baurii* varieties (Table 7a). Nine out of 12 individuals of *R. baurii* var. *baurii* from Hebron Farm were diploid and the remaining three individuals were tetraploid. Of the *R. baurii* var. *platypetala* individuals from Hebron Farm there were two diploid individuals and 11 tetraploid individuals. Of the *R. baurii* var. *platypetala* at Sunset Farm, there were two diploid individuals, one triploid individual, as well as one tetraploid individual. All 15 analysed *R. milloides* individuals from Sunset Farm were tetraploid.

Table 7a. Ploidy estimates for *R. baurii* varieties and *R. milloides* that occur at the two focal populations. The cytotyped plants were used as maternal or paternal individuals in the crosses.

Taxon (total N)	Population	Diploid 2x	Triploid 3x	Tetraploid 4x
R. baurii var. *platypetala* (n=4)	Sunset Farm	2	1	1
R. baurii var. *platypetala* (n=13)	Hebron Farm	2	0	11
R. baurii var. *baurii* (n=12)	Hebron Farm	9	0	3
R. milloides (n=10)	Sunset Farm	0	0	10

Table 7b. Ploidy levels and 2C DNA content in picograms for *Rhodohypoxis* hybrids that were made during this experiment. SM = *R. milloides* from Sunset Farm, BH = *R. baurii* var. *baurii* from Hebron Farm, PH = *R. baurii* var. *platypetala* from Hebron Farm**.** There was only one individual plant cytotyped per hybrid cross and each cross was only performed once between the maternal and paternal plants. Estimates were done using *Pisum sativa* as the internal standard.

Maternal ID	Paternal ID	2C DNA content (pg)	Ploidy
SM15	PH12	3.85	4x
BH8	CS6	2.00	2x
PS3	BH12	1.68	2x
SM12	PH3	3.98	4x
SM7	PH12	3.93	4x
SM2	PH10	3.98	4x
SM5	PH10	4.06	4x
PH3	SM12	4.28	4x
PH14	PS11	2.00	2x

CHAPTER 4: DISCUSSION

4.1. Discussion

The main aim of this study was to test for the presence of postzygotic reproductive isolation among *Rhodohypoxis baurii* varieties and between *R. baurii* and *R. milloides*. There were three objectives: 1) to examine postzygotic barriers through controlled crossing experiments, 2) to characterize gene flow among *R. baurii* varieties and between R. baurii and *R. milloides*, and 3) to estimate ploidy levels for each *R. baurii* variety and *R. milloides* and resulting hybrid offspring.

Reproductive barriers may act sequentially to limit gene flow among closely related taxa (Dobzhansky, 1937; Mayr, 1942; Muller, 1940; Ramsey *et al.*, 2003) eventually leading to species divergence. Because they often act by first preventing gene flow, prezygotic barriers are often regarded as being the most influential in decreasing interspecific gene flow (Ramsey *et al.*, 2003; Moyle *et al.*, 2004; Kay, 2006; Lowry, 2008). Shifts in pollinator preference and phenology are often the strongest prezygotic barriers that lead to speciation (Martin and Willis, 2007). Complementary to prezygotic barriers are postzygotic barriers that occur after fertilization and are mainly under genetic control (Coyne and Orr, 1989; Coyne and Orr, 2004). In the event that prezygotic barriers have not yet had the strength to prevent successful reproduction, postzygotic barriers may form first and strongly influence the course of speciation in taxa (Widmer *et al.*, 2009). Polyploidy can also influence speciation in taxa by acting as a postzygotic barrier between taxa of different ploidies during hybridization.

Pre- and post-zygotic barriers

The three *Rhodohypoxis baurii* varieties were observed to occur in different ecological habitats, which were consistent with habitats described by Hilliard and Burtt (1978). *R. baurii* var. *baurii* is found in moist, shaded areas at the base of cliff faces, *R. baurii* var. *platypetala* prefers dry, rocky soils, and *R. baurii* var. *confecta* is found in habitats that

are a combination of rocky soils and moist, shaded areas. *R. milloides* is found in marshy areas. In their 1978 compilation of southern African plants, Hilliard and Burtt (1978 p. 48) described the ecological differences between the three *R. baurii* varieties as being "distinct, yet subtle". The differences in habitat preferences among the *R. baurii* varieties and *R. milloides* indicate the presence of ecogeographic isolation (per Sobel *et al*., 2010). Ecological adaptations are major drivers of reproductive isolation (Schluter, 2009; Sobel *et al.*, 2010) because adaptations to different environments may limit gene flow between populations. Ecological or habitat shifts have likely played a role in the diversification in *Rhodohypoxis* due to habitat heterogeneity. The diversity of microhabitats available in the native range of these taxa has been closely linked to tectonic uplift events in the Drakensberg that occurred during the Pliocene (5 Ma; King and King, 1959; Bentley *et al.*, 2014). In the Drakensberg near-endemic genus *Macowania* Oliv., it is estimated that diversification occurred following the Pliocene tectonic uplift that catalyzed speciation due to ecological and geographical niches that became available (Bentley *et al.*, 2014). Previous work towards resolving relationships among *Rhodohypoxis* species has suggested that the genus is recently diverged (Uys and Cron, unpublished data). When coupled with differences in geography, habitat preference could potentially be a form of prezygotic isolation in *Rhodohypoxis baurii* varieties and *R. milloides*.

The flowering times of *R. baurii* and *R. milloides* were found to overlap considerably with differences only occurring between the dates of initial flowering in the greenhouse during the 2018 flowering season. *R. baurii* var. *baurii* flowers first in late August, followed by *R. baurii* var. *platypetala* in the first week of September, *R. baurii* var. *confecta* flowers late in September, and *R. milloides* may start flowering as late as the last week of October. The three taxa then continue to produce flowers until the end of the season, in February (Pers. Obs.). This observation was made in the field whilst collecting plants. As the phenology data were collected under greenhouse conditions, the actual phenology in the natural environment

might not be well reflected. In their description of *Rhodohypoxis,* Hilliard and Burtt (1978) described flowering times for the three varieties as ranging from early November through December, but do not give precise accounts of flowering time for each of the varieties. Personal observations in the field indicate that flowering starts from as early as October in *R. baurii* var. *confecta,* and continues until mid December. It is possible that the *Rhodohypoxis* plants in the greenhouse have acclimatized to greenhouse conditions, and now have similar flowering times. Other studies that have compared plants that have been grown under controlled environments to those that have been grown in the field, have often done so when comparing phenotypic traits, and have not necessarily been related to phenological responses (Poorter, *et al*., 2016). In one study where the phenological development of *Brassica* L. species in a controlled environment was compared to that of *Brassica* species in their natural environment, it was found that the plants in the controlled environment flowered earlier than those in their natural environment (Nanda *et al*., 1996). If the greenhouse phenology reflects that of the natural flowering times, then phenology is a relatively weak prezygotic barrier because it does not decrease/prevent gene flow between the taxa.

Pollinators have not yet been observed for *Rhodohypoxis*. Studies are currently underway to try to identify potential pollinators. This should help shed light on the prevalence of prezygotic barriers since phenology and pollinator specificity are often the strongest prezygotic barriers (Ramsey *et al.,* 2003). This lack of phenological barrier is similar to the sympatric species *Chamaecrista desvauxii* var. *graminea* H. S Irwin & Barneby (Fabaceae) and *C. desvauxii* var. *latistipula* (Benth.) G. P. Lewis where prezygotic barriers are weak or absent between these two varieties due to overlapping flowering periods, as well sharing the same pollinator, *Bombus brevivillus* Franklin (Costa *et al.,* 2007). The absence or weakness of prezygotic barriers may mean that postzygotic barriers may have a more important evolutionary role in these taxa. The prevalence of postzygotic barriers between the *Chamaecrista desvauxii* (Collad.) Killip varieties is supported by the fact that in the absence

of prezygotic barriers, postzygotic barriers arise first in the form of seed abortion (Costa *et al.*, 2007). In a study on reproductive isolation in Mediterranean orchids (Orchidinae), postzygotic barriers were found to play a more prominent evolutionary role than prezygotic barriers (Cozzolino *et al*., 2004). An overlap in pollinators in these orchid species renders pollinator isolation a weak prezygotic barrier. Orchids that share pollinators display karyotypes that are structurally different from karyotypes in orchids with specialized pollinators (Cozzolino *et al.*, 2004). This divergence in karyotypes may indicate that postzygotic reproductive barriers played a more influential role in the evolution of the orchids than prezygotic barriers (Cozzolino *et al.*, 2004). The perceived absence of pollinators could therefore indicate that levels of outcrossing within *Rhodohypoxis* are low and that selfing is the dominant form of reproduction.

Differences in mating systems may also contribute to prezygotic reproductive isolation. Self-compatibility was tested through autogamous hand-crosses for each variety. All of the varieties were found to be self-compatible, as well as possessing the ability to cross-pollinate between varieties and with *R. milloides*. Flowers that were autogamously selfed collectively produced a higher average number of seeds than either intravarietal or intraspecific crosses (Figure 4). Interestingly, intravarietal crosses produced fewer seeds than intervarietal crosses (Figure 4). Seed production among and between varieties confirms that the varieties are all the same species under the Biological Species Concept. This result may be due in part to there being a higher number of intervarietal crosses than there were intravarietal crosses. The disparity between the numbers of crosses can be accounted for by the availability of flowers to cross during the flowering season. Intervarietal and interspecific crosses were prioritized for experimental purposes. More data are still needed to confirm whether self-pollination can occur autonomously in nature in *Rhodohypoxis*. If spontaneous autogamous self-pollination occurs naturally, then self-compatibility could be a possible prezygotic barrier. Self-compatibility is commonly seen in recently diverged lineages and

may evolve as a result of selection in response to ecological shifts (Barret and Eckert, 1990). This may be applicable to *Rhodohypoxis* because the genus has recently diverged (Uys and Cron, unpublished data). Self-compatibility can also arise as a form of reproductive assurance in areas where pollinators are scarce or are unpredictable (Baker, 1955; Stebbins, 1957). Unpredictable weather in mountainous areas, in which the *R. baurii* occur, can contribute to the scarcity of pollinators because it may restrict or disrupt the flight activity of pollinators, which in turn decreases flower visitation rates (McCall and Primack, 1992). This limitation in pollinators in mountainous areas has also been linked to higher rates of apomixis in mountain/alpine flowering populations (Arroyo *et al*, 2006). Preliminary greenhouse and field experiments to test for apomixis in the *R. baurii* varieties have so far shown that they are not apomictic. Changes in ploidy have also been attributed to self-compatibility (Stebbins, 1950; Mable, 2004; Barringer, 2007), however since both diploid and tetraploid varieties are self-compatible, it is unlikely that ploidy has driven self-compatibility in these species/varieties of *Rhodohypoxis*.

The *Rhodohypoxis baurii* varieties are also capable of outcrossing because they produce viable seeds when crossed with a different *R. baurii* variety. The observed self-compatibility within *R. baurii* and the varieties' ability to outcross presents the challenge of determining outcrossing and selfing rates to test whether one mating system is more prevalent than the other, or whether *R. baurii* has a mixed mating system (most likely). Determining the predominant mating system has implications with regards to determining the likelihood of hybridization (Grant, 1981; Rieseberg, 1997), as well as providing a better understanding in the distribution of genetic variation within and among populations and individuals (Ludwig *et al.*, 2013; Pickup *et al.*, 2019). Further, the floral architecture of *Rhodohypoxis* is similar to that of self-compatible species in that the flowers comprise small, closed petals, and reproductive organs that are in close contact (Barrett, 2010). To date, there is no evidence for

nectar production associated with *Rhodohypoxis* (pers. obs.), which could support potential self-compatibility.

The success of crosses in the greenhouse is also a possible indicator of hybridization occurring in natural populations of *Rhodohypoxis*. In their 1978 account of putative hybrids, Hilliard and Burtt described hybrids as being in environments that were intermediate to those of their parents, meaning that a hybrid between *R. baurii* var. *baurii* and *R. baurii* var. *platypetala* would be found in an area that was an intermediary habitat between dry, rocky soils and a wet habitat. Putative hybrids between *R. baurii* var. *baurii* and *R. baurii* var. *platypetala* were found in what was described as a narrow hybrid zone on Hebron Farm and in the Zuurberg Mountains (Hilliard and Burtt, 1978). Hybrids between *R. baurii* var. *platypetala* and *R. milloides* were found on Sunset Farm as well as in the Giant's Castle Nature reserve (Hilliard and Burtt, 1978). Similarly, the hybrid would have intermediate morphological features that would be a combination of the parents' morphological features. Hilliard and Burtt (1978) described hybrids between *R. milloides* and *R. baurii* var. *platypetala* as having pink flowers. This description of putative hybrids between *R. baurii* var. *platypetala* and *R. milloides* is similar to the hybrids obtained in greenhouse crosses between *R. milloides* and *R. baurii* var. *platypetala*. The hybrid had light pink petals, a combination of the magenta and white of its parental species (Figure 8a).

Hybrid seed inviability

Asymmetrical seed production

In an attempt to test whether maternal identity affects seed production, a generalized linear model was used. I found that when *R. baurii* var. *baurii* is crossed with *R. milloides* or with *R. baurii* var. *confecta* there is a significant difference in seed production compared to when *R. baurii* var. *baurii* is crossed with either *R. baurii* var. *platypetala* or with other *R. baurii* var. *baurii* individuals. The maternal identity of *R. baurii* varieties did not affect seed

production when they were crossed with each other or with *R. milloides.* Results obtained from the GLM contradict results on seed set where there appears to be asymmetry in seed production in crosses that have *R. baurii* var. *platypetala* as the maternal plant (Table 3). Asymmetries in postzygotic isolation often occur as a result of nuclear-cytoplasm interactions (Tiffin *et al.,* 2001), which are a type of DMIs. This result implies that there may be maternal effects affecting seed production in crosses where *R. baurii* var. *baurii* when crossed with individuals of *R. milloides* or *R. baurii* var. *confecta,* and that the maternal effects are asymmetrical. Maternal effects are a result of a parental genomic imbalance whereby one cytotype contributes more to the genome than the other cytotype. In between-ploidy crosses, maternal effects are defined as being the influence of the maternal genotype or phenotype on the offspring genotype or phenotype (Wolf and Wade, 2009).

Germination

On average, 47.25% of seeds produced across the different cross types germinated successfully. Interestingly, interspecific seeds where *R. milloides* was the maternal taxon had the highest germination percentage (68.6%) relative to other cross types. Intraspecific crosses had the second highest germination percentage where 42.86% of the seeds germinated. Intra-varietal crosses had an average percentage germination of 39.13%, whilst inter-varietal crosses had a germination percentage of 38.14%. If the three *R. baurii* varieties are independent evolutionary lineages, one would expect intravarietal seeds to have a higher proportion of viable seeds than intervarietal seeds. The difference in germination between intervarietal and intravarietal crosses is small, however when compared to the average percentage germination (germination rate that includes all taxa that were planted and germinated) (47.25%), both germination values for intervarietal (38.14%) and intravarietal (39.13%) are quite low. A surprising result is that interspecific crosses (68.86 %) exceeded

the average percentage germination value (47.25%), as well as the intraspecific germination value (42.86%).

Assessing seed viability through germination is important because of the direct effect that germination has on local adaptation and natural selection (Clausen and Hiesey, 1940; Hämälä *et al.*, 2017). *Rhodohypoxis baurii* varieties are found in different habitats. These differences in habitats may apply a selective pressure on hybrid seeds that could render them inviable and unable to germinate in the parental environments. Germinating the varieties in a greenhouse environment, where everything is controlled, may give us an overestimate of the actual proportion of germination that occurs in natural hybridizing populations. The overestimation of germination proportions is potentially even more probable by the fact that the varieties naturally display markedly different ecological preferences. Successful germination and survival of hybrid seedlings would likely have to occur in conditions that are a combination of the parental taxa's preferred natural environment instead of in a controlled environment where conditions are uniform. From the observed low germination percentages between the three *R. baurii* varieties, one may conclude that seed inviability in the form of germination failure, is incipient and is in its early stages. In crosses between two populations of subspecies of *Arabidopsis lyrata* (L.) O'Kane & Al-Shehbaz; *A. lyrata* ssp. *petrea* (L.) O'Kane & Al-Shehbaz and *A. lyrata* ssp. *lyrata* (L.) O'Kane & Al-Shehbaz, F_1 hybrids had a lower germination proportion than seeds of parental crosses (Hämälä *et al.*, 2017). The decreased proportion of germinated hybrids was attributed to endosperm defects that occur when there is a deviation from the 2:1 maternal to paternal genome ratio required for normal endosperm development (Ramsey and Schemske, 1998). Endosperm defects were not tested for in this project, but should be considered for future research.

Germination success is also frequently affected by differences in ploidy between crossing taxa (Ramsey and Schemske, 1998; Sutherland and Galloway, 2017). In diploid–

diploid crosses, hybrid seed inviability is mostly decreased by DMIs, whilst in interploid crosses, endosperm defects known as the triploid block are the most likely to cause seed inviability (Ramsey and Schemske, 1998). The ploidies of *Rhodohypoxis* individuals used in this study were determined through flow cytometry. We found that *R. baurii* var. *baurii* and *R. baurii* var. *confecta* individuals used in this study are both primarily diploid, and that *R. baurii* var. *platypetala* and *R. milloides* individuals used in this study are tetraploid (Table 7a). If differences in ploidy affect postzygotic isolation within the Sunset and Hebron Farm populations, then postzygotic isolation most likely to be caused by the triploid block that occurs as a result of a parental genomic imbalance (e.g., Baack, 2005; Köhler *et al.*, 2010). In diploid-diploid crosses, the ratio of maternal to paternal genome contribution is 2:1 to form a triploid endosperm during the process of double fertilization. Interploid crosses deviate from this ratio and the deviation results in defects in the endosperm, which may lead to seed inviability and germination failure (Ramsey and Schemske, 1998, Köhler, 2010). In diploid-tetraploid crosses between *Campanula rotundifolia* L. (Campanulaceae) Sutherland and Galloway (2017) found that germination was significantly reduced compared to intraploid crosses. This reduction in germination was attributed to triploid block caused by parental genomic imbalances. Unlike interploid crosses in *Campanula*, in interploid crosses between *Rhodohypoxis* varieties, germination was not reduced as would be expected with an interploid cross. It is therefore unlikely that postzygotic isolation could be caused by triploid block or endosperm defects in *Rhodohypoxis.*

Two of the germinated hybrid seedlings flowered, one in 2018 and one hybrid in 2019. Both seeds were the result of reciprocal crosses between the same individuals; *R. milloides* from Sunset Farm and *R. baurii* var. *platypetala* from Hebron Farm. Both hybrid flowers were a light pink colour, which is intermediate to that of the parent plants. In their description of putative hybrids between *R. milloides* and *R. baurii* var. *platypetala*, Hilliard and Burtt (1978) described the hybrid flowers as being pink in colour, similar to the hybrids

observed in the greenhouse. Interestingly, differences in the petals of the two hybrid flowers were observed. The hybrid that had an *R. baurii* var. *platypetala* as a maternal plant had the typical compact *R. baurii* petal shape. The hybrid that had *R. milloides* as a maternal plant had elongated petals that appeared folded. The petals of the flower were not compact, and the anthers were visible, unlike in typical *R. baurii* flowers where petals need to be pulled apart in order to reveal the anthers. The deformation of petals may be caused by a genetic defect during floral development (Bradshaw and Schemske, 2003), which could possibly render the hybrid extrinsically inviable in its environment (Coyne and Orr 1998).

A preliminary examination of pollen viability was conducted on hybrid pollen and on *R. baurii* var. *baurii*. An F_1 hybrid between *R. milloides* and *R. baurii* var. *platypetala* had pollen that was mostly inviable, with 43.75% of the pollen being viable. The inviability of the pollen suggests that hybrids are not entirely fertile, and that pollen inviability may be a potential postzygotic barrier that would increase exponentially in subsequent hybrid generations (Coyne and Orr, 1989). To ascertain pollen inviability as a potential postzygotic barrier between *Rhodohypoxis* taxa, further crossing experiments would need to be done with F_1 hybrids to assess pollen viability in F_2 progeny.

Population genetic variation

Population genetic structure gives an indication of the distribution of alleles and genetic diversity within populations. The mean genetic diversity at both Hebron farm (0.385) and Sunset Farm (0.420) was low, which suggests there is little genetic differentiation within the populations. Low genetic differentiation is further supported by the low genetic distances between the varieties, with the greatest genetic distance being between *R. baurii* var. *baurii* from Hebron Farm and *R. milloides* from Sunset Farm. This is as expected because *R. baurii* var. *baurii* and *R. milloides* are different species from separate localities.

Population structure results indicate admixture in both the Sunset Farm and Hebron Farm populations, with Sunset Farm showing the most admixture (Figure 6a). This admixture is indicative of frequent past hybridization, possibly repeated backcrossing within the genus, which would result in ancestral polymorphisms. Notably, some individuals of *R. milloides* share the majority of their genotype with *R. baurii* var. *platypetala*. In their accounts of *R. milloides* populations, Hilliard and Burtt (1978) mention that *R. baurii* var. *platypetala* plants that appear to have been introgressed by *R. milloides* based on morphology. The flowers on these plants are described as having a deep red colour and were found growing in the rocky *R. baurii* var. *platypetala* habitat. Introgression among closely related plant species is relatively common. For example, Martinsen *et al.* (2001) found that introgression occurs in between two species of cottonwood trees (*Populus fremontii* S. Wats and *P. angustifolia* James), and that the introgression between those species may be adaptive because of the transferal of genes that increase fitness in. Introgression has also been shown to occur between the sunflowers *Helianthus annuus* and *H. petiolaris* (Yatabe *et al.*, 2007), despite strong reproductive barriers such as differences in edaphic preferences.

Introgression may increase genetic variation in a population or it can cause genes of one species to become dominant in the population (Stebbins, 1971). It is often challenging to distinguish between ancestral polymorphisms and introgression. If introgression was adaptive, then we would likely see increased genetic diversity (Rieseberg *et al*., 1993). This is not the case with *Rhodohypoxis* in either the Sunset or the Hebron population, as genetic diversity values were low in both populations. The ease with which hybrids were formed in the greenhouse supports the likelihood of introgression having occurred in the taxa's natural habitat. The ease with which hybrids formed also points to reproductive barriers being weak between *R. baurii* varieties and *R. milloides.* Genomic regions with reduced opportunities for introgression are often associated with loci that strongly contribute to reproductive isolation (Larson *et al.,* 2013). This implies that in populations with high levels of introgression there

are not many genes that actively contribute to reproductive isolation, and thus reproductive isolation will not be strong. This could be the case among *Rhodohypoxis baurii* varieties and *R. milloides*. If genetic (intrinsic) factors that contribute to reproductive isolation are absent or not expressed, then the varieties cannot be classified as different species. Further, these intrinsic factors can influence both prezygotic and postzygotic isolation. Factors that can contribute to reproductive isolation are often under genetic control (Bradshaw and Schemske, 2003; flower colour, petal shape and size).

In the Sunset farm populations, introgression could be facilitated by ploidy because *R. milloides* and *R. baurii* var. *platypetala* are both tetraploids. In Hebron, introgression may occur despite the differences in ploidy between *R. baurii* var. *baurii* (2x) and *R. baurii* var. *platypetala* (4x). This could be as a result of triploid bridge formation within the population, but we have not sampled deeply enough to test this hypothesis. There are few examples of introgression occurring across ploidy levels in natural populations. One such example is that of introgression between *Senecio vulgaris* L. (2x) and *S. squalidus* Bieb. (4x). Genes from *S. squalidus* were introgressed into *S. vulgaris*, and resulted in the origin of a new variety of *S. vulgaris* that was more attractive to pollinators (Chapman and Abbott, 2010). Another example of where introgression has occurred across a ploidy level is between *Capsella rubella* Reut. (2x, Brassicaceae) and *C. bursa-pastoris* (Reut.) Hobk (4x; Slotte *et al.,* 2008). These two species may bear similarities to *Rhodohypoxis* because they may also predominantly self and have slight differences in flowering time.

4.2. Conclusion

I sought to test for the presence of postzygotic reproductive isolation among *R. baurii* varieties and between *R. baurii* varieties and *R. milloides*. From the results it can be concluded that there are potentially few prezygotic barriers between any of these taxa. Habitat differences have the potential to be strong selection agents in the divergence of *R. baurii* since all three varieties have distinct habitats. Phenology as a prezygotic barrier proved

to be weak, due to the overlap in flowering times. The pollinators of *R. baurii* remain unknown and are the focus of ongoing research. More information on pollinators would help determine the frequency of selfing versus outcrossing within the genus. Early onset intrinsic postzygotic barriers such as seed inviability do not appear to be strong, as some hybrid seeds are viable (47.25% of hybrid seeds germinated on average). There is strong potential for the inviability of future generations of hybrids through pollen inviability and extrinsic hybrid inviability. A thorough investigation of postzygotic barriers would require future studies to generate F_2 hybrids to test viability, as postzygotic isolation may manifest stronger in further generations. The extent of hybridization and introgression within the genus has the potential to be important in the evolutionary trajectory of the species, and could either increase allelic diversity, or lead to a genetic swamp. Despite having found differences in ploidy between the three varieties and *R. milloides*, differences in ploidy does not seem to play a role in postzygotic isolation as it did not appear to affect seed germination. Further ploidy studies should aim to determine whether *R. baurii* var. *platypetala* and *R. milloides* are autopolyploids or allopolyploids, as this can aid in better understanding the history of hybridization within the genus. Habitat differences coupled with the potential for hybrid pollen inviability as well as extrinsic hybrid inviability, have potential to be the driving isolating barriers in the speciation of *R. baurii* and *R. milloides*.

References

Abbott, R., Albach, D., Ansell, S., Arntzen, J.W., Baird, S.J.E., Bierne, N., Boughman, J., Brelsford, A., Buerkle, C.A., Buggs, R., Butlin, R.K., Dieckmann, U., Eroukhmanoff, F., Grill, A., Cahan, S.H., Hermansen, J.S., Hewitt, G., Hudson, A.G., Jiggins, C., Jones, J., Keller, B., Marczewski, T., Mallet, J., Martinez-Rodriguez, P., Möst, M., Mullen, S., Nichols, R., Nolte, A.W., Parisod, C., Pfennig, K., Rice, A.M., Ritchie, M.G., Seifert, B., Smadja, C.M., Stelkens, R., Szymura, J.M., Väinölä, R., Wolf, J.B.W., Zinner, D. 2013. Hybridization and speciation. *Journal of Evolutionary Biology* **26**: 229-246.

Anderson, B., Alexandersson, R., and Johnson, S.D. 2010. Evolution and coexistence of pollination ecotypes in an African *Gladiolus* (Iridaceae). *Evolution* **64**: 960-972.

Arnold, M.L. 2006. *Evolution through genetic exchange*. Oxford University Press, New York.

Arroyo, M.T.K., Munoz, S.M., Henríquez, C., Till-Bottraud, I., and Pérez, F. 2006. Erratic pollination, high selfing levels and their correlates and consequences in an altitudinally widespread above-tree-line species in the high Andes of Chile. *Acta Oecologica* **30**: 248-257.

Baack, E.J. 2005. T succeed globally, disperse locally: effects of local pollen and seed dispersal on tetraploid establishment. *Heredity* **94**: 538-546.

Baker, H.G. 1955. Self-compatibility and establishment after "long-distance" dispersal. *Evolution* **9**: 347-349.

Ballard, J.W., and Rand, D.M. 2005. The population biology of mitochondrial DNA and its phylogenetic implications. *Annual Review of Ecology, Evolution, and Systematics* **36**: 621-642.

Barnard-Kubow, K.B, and Galloway, L.F. 2017. Variation in reproductive isolation across a species range. *Ecology and Evolution* **7**: 9347-9357.

Barton, N.H., and Hewitt, G.M. 1985. Analysis of Hybrid Zones. *Annual Review of Ecology and Systematics* **16**: 113-148.

Barton, N.H. 2001. The role of hybridization in evolution. *Molecular Ecology* **10**: 551-568.

Barrett, S.C.H., and Eckert, C.G. 1990. Current issues in plant reproductive ecology. *Israel Journal of Botany* **39**: 5-12.

Barrett, S.C.H. 2010. Understanding plant reproductive diversity. *Philosophical Transactions of the Royal Society: Biological Sciences* **365**: 99-109.

Barringer, B. 2007. Polyploidy and self-fertilization in flowering plants. *American Journal of Botany* **94**: 152-1533.

Barringer, B., and Geber, M.A. 2008. Mating system and ploidy influence levels of inbreeding depression in *Clarkia* (Onagraceae). *Evolution* **62**: 1040-1051.

Bates, D., Machler, M., Bolker, B., and Walker, S. 2015. Fitting Linear Mixed-Effects Models Using lme4. *Journal of Statistical Software* **67**:1-48.

Bateson, W. 1909. Heredity and variation in modern lights in *Darwin and Modern Science*, edited by A.C. Seward. Cambridge University Press, Cambridge.

Bensch, S., and Åkesson, M. 2007. Ten years of AFLP in ecology and evolution: why so few animals? *Molecular Ecology* **14**: 2899-2914.

Bentley, J., Verboom, A.G., and Bergh, N.G. 2014. Erosive processes after tectonic uplift stimulate vicariant and adaptive speciation: evolution in an Afrotemperate-endemic paper daisy genus. *BMC Evolutionary Biology* **14**: 1-16.

Blears, M. J., De Grandis, S. A., Lee, H., and Trevors, J. T. 1998. Amplified fragment length polymorphism (AFLP): A review of the procedure and its applications. *Journal of Industrial Microbiology and Biotechnology* **21**: 99-114

Blignaut, M., Ellis, A.G., and Le Roux, J.J. 2013. Towards a Transferable and Cost-Effective Plant AFLP Protocol. *Public Library of Science ONE* **8**: 1-7.

Bomblies, K., Lempe, J., Epple, P., Warthmann, N., Lanz, C., Danlg, J.L., and Weigl, D. 2007. Autoimmune Respone as a Mechanism for a Dobzhansky-Muller-Type Incompatibility in Plants. *Public Library of Science Biology* **5**: e263

Bradshaw, H.D., and Schemske, D.W. 2003. Allele substitution at a flower colour locus produces a pollinator shift in monkeyflowers. *Nature* **426**: 176-178.

Chapman, M.A., and Abbott, R.J. 2010. Introgression of fitness genes across a ploidy barrier. *New Phytologist* **186**: 63-71.

Clausen, J., and Hiesey, W.M. 1940. *Experimental Studies on the Nature of Species, Volume 1: Effect of Varied Environments on Western North American Plants 520.* Carnegie Institution of Washington, Washington DC.

Cook, L.M., and Soltis, P.S. 1999. Mating systems of diploid and allotetraploid populations of *Tragopogon* (Asteraceae). I. Natural populations. *Heredity* **82**: 237-244.

Costa, C. B.N., Lambert, S. M., Borba, E. L., and De Queiroz, L. P. 2007. Post-Zygotic Reproductive Isolation between Sympatric Taxa in the *Chamaecrista desvauxii* Complex (Leguminosae-Caesalpinioideae). *Annals of Botany* **99**: 625-635.

Costa, R., Pereira, G., Garrido, I., Tvares-de-Sousa, M.M., and Espinosa, F. 2016. Comparison of RAPD, ISSR, and AFLP Molecular Markers to Reveal and Classify Orchardgrass (*Dactylis glomerata* L.) Germplasm Variations. *PLoS One* **11**: e0152972.

Coyne, J.A and Orr, H.A. 1989. Patterns of Speciation in *Drosophila*. *Evolution* **43**: 362-381.

Coyne, J.A and Orr, H.A. 1998. The evolutionary genetics of speciation. *Philosophical Transactions of the Royal Society of London B: Biological Sciences* **353**: 287-305.

Coyne, J.A and Orr, H.A. 2004. *Speciation*. Sinauer. Chicago.

Cozzolino, S., D'Emerico, S., and Widmer, A. 2004. Evidence for reproductive isolate selection in Mediterranean orchids: karyotype differences compensate for the lack of pollinator specificity. *Proceedings of the Royal Society of London: Series B: Biological Sciences* **271**: 259-262.

Cruzan, M.B., and Arnold, M.L. 1994. Assortative mating and natural selection in an *Iris* hybrid zone. *Evolution* **48**: 1946-1958.

Darwin, C., R. 1859. On the Origin of Species by Means of Natural Selection, or, the Preservation of Favoured Races in the Struggle for Life. John Murray. London.

De Queiroz, K. 2005. Different species problems and their resolution. *BioEssays* **27**: 1263-1269

Dell'Olivo, A., Hoballah, M. E., Gübitz, T., and Kuhlemeier, C. 2011. Isolation barriers between *Petunia axillaris* and *Petunia integrifolia* (Solanaceae). *Evolution* **65**: 1979-1991.

Dobzhansky, T. 1935. A critique of the species concept in biology. *Philosophy of Science* **2**: 344-355.

Dobzhansky, T. 1937. *Genetics and the Origin of Species*. Colombia University Press. New York.

Dolezel, J., Greilhuber, J., Lucretti, S., Meister, M. A., Lysak, A., Nardi, L., and Obermayer, R. 1998. Plant Genome Size Estimation by Flow Cytometry: Inter-laboratory Comparison. *Annals of Botany* **82**:17-26.

Doyle, J.J., and Doyle, J.L. 1987. A rapid DNA isolation procedure for small quantities of fresh leaf tissue. *Phytochemical Bulletin* **19**: 11-15.

Ellis, A.G., Weis, A.E., and Gaut, B.S. 2007. Evolutionary radiation of "stone plants" in the genus *Argyroderma* (Aizoaceae): Unravelling the effects of landscape, habitat, and flowering time. *Evolution* **60**: 39-55.

Elzinga, J.A., Atlan, A., Biere, A., Gigord, L., Weis, A.E., and Bernasconi, G. Time after time: flowering phenology and biotic interactions. 2007. *Trends in Ecology & Evolution* **22**: 432-439.

Falush, D., Stephens, M., and Pritchard, J.K. 2003a. Traces of Human Migrations in *Helicobacter pylori* Populations. Populations. *Science* **299**: 1582-1585.

Falush, D., Stephens, M., and Pritchard, J.K. 2003b. Inference of population structure using multilocus genotype data: linked loci and correlated allele frequencies. *Genetics* **164**: 1567-1587.

Falush, D., Stephens, M., and Pritchard, J.K. 2007. Inference of population structure using multilocus genotype data: dominant markers and null alleles. *Molecular Ecology Notes* **7**: 574-578.

Glennon, K.L., Rissler, L.J., and Church, S.A. Ecogeographic isolation: a reproductive barrier between species and cytotypes in *Houstonia* (Rubiaceae). 2012. *Evolutionary Ecology* **26**: 909-926.

Grant, V. 1981. *Plant Speciation 2nd edition*. Columbia University Press, New York.

Grant, R.P and Grant, B.R. 1992. Hybridization of bird species. *Science* **256**: 193-197.

Grant, V. 1993. Origin of floral isolation between ornithophilous and sphingophilous plant species. *Proceedings of the National Academy of Sciences* **90**: 7729-7733.

Grant, R, B and Grant, P.R. 1997. Genetics and the origin of bird species. *Proceedings of the National Academy of Sciences* **94**: 7768-7775.

Gregory, R.T. 2004. Insertion-deletion biases and the evolution of genome size. *Genetics* **324**: 15-34.

Hämälä, T., Mattila, T.M., Leinonen, P.H., Kuittinen, H., and Savolainen, O. 2017. *Molecular Ecology* **26**: 3484-3496.

Hannweg, K., Sippel, A., and Bertling, I. 2013. A simple and effective method for the micropropagation an *in vitro* induction of polyploidy and the effect on floral characteristics of the South African iris, *Crocosmia aurea*. *South African Journal of Botany* **88**: 367-372.

Hatfield, T., and Schluter, D. 1999. Ecological Speciation in Sticlebacks: Environment-Dependent Hybrid Fitness. *Evolution* **53**: 866-873.

Hersch-Greene, E.I. 2012. Polyploidy in Indian paintbrush (*Castilleja*; Orobancjaceae) species shapes but does not prevent gene flow across species boundaries. *American Journal of Botany* **99**: 1680-1690.

Hey, J., Waples, R.S., Arnold, L.M., Butlin, R.K., and Harrison, R.G. 2003. Understanding and confronting species uncertainty in biology and conservation. *Trends in Ecology & Evolution* **18**: 597-603.

Hiesey, W., Nobs, M., and Bjorkman, O. 1971. *Experimental studies on the nature of species. V. Biosytematics, Genetics, and Physiological Ecology of the* Erythranthe *Section of* Mimulus. Carnegie Institute. Washington DC.

Hijmans, R.J., Gavrilenko, T., Stephenson, S., Bamberg, J., Salas, A., and Spooner, D.M. 2007. Geographical and environmental range expansion through polyploidy in wild potatoes (*Solanum* section *Petota*). *Global Ecology and Biogeography* **16**: 485-495.

Hilliard, O.M and Burtt, B.L. 1978. Notes on some plants from Southern Africa chiefly from Natal: VII. *Notes from the Royal Botanic Garden Edinburgh* **36**: 43-65.

Husband, B.C. 2000. Constraints on polyploidy evolution: a test of the minority cytotype exclusion principle. *Proceedings of the Royal Society: Biological Sciences* **267**: 217-223.

Husband, B.C and Schemske, D.W. 2001. Ecological mechanisms of reproductive isolation between diploid and tetraploid *Chamerion angustifolium*. *Journal of Ecology* **88**: 689–701.of southern African flora. *Philosophical Transactions of the Royal Society B* **365**: 499-516.

Husband, B.C., Ozimec, B., Martin, S.L, and Pollock, L. 2008. Mating consequences of polyploidy evolution in flowering plants: current trends and insights from synthetic polyploids. *International Journal of Plant Sciences* **169**: 195-206.

Johnson, S.D. 2010. The pollination niche and its role in the diversification and maintenance of the southern African flora. *Philosophical Transactions of the Royal Society B Biological Sciences* **365**: 499-516.

Kay, K.M. 2006. Reproductive isolation between two closely related hummingbird pollinated neotropical gingers. *Evolution* **60**: 538-552.

Kearns, C.A and Inouye, D.W. 1993. Techniques for Pollination Biologists. University Press of Colorado. Niwot, CO. 77-151.

King, L.C., and King, L.A. 1959. A Reappraisal of the Natal Monocline. *South African Journal Geographical Journal* **41**: 15-30.

Köhler, C., Scheid, O.M., and Erilova, A. 2010. The impact of triploid block on the origin and evolution of polyploidy plants. *Trends in Genetics* **26**: 142-148.

Kozak, G.M., Rudolph, A.B., Colon, B.L., and Fuller, R.C. 2012. Postzygotic Isolation Evolves before Prezygotic Isolation between Fresh and Saltwater Populations of the

Rainwater Killifish, *Lucania parva*. *International Journal of Evolutionary Biology* **2012**: 1-11.

Krejčíková, J., Sudová, R., Lučanová, M., Trávníček, P., Urfus, T., Vít, P., Weiss-Schneeweiss, H., Kolano, B., Oberlander, K., Dreyer, L.L., and Suda, J. 2013. High ploidy diversity and distinct patterns of cytotype distribution in a widespread species of *Oxalis* in the Greater Cape Floristic Region. *Annals of Botany* **111**: 641-649.

Laffon-Placette, C., and Köhler, C. 2016. Endosperm – based postzygotic hybridization barriers: developmental mechanisms and evolutionary drivers. *Molecular Ecology* **25**: 2620-2629.

Larson, E. L., White, T.A., Ross, C.L., and Harrison, R. G. 2013. Gene flow and the maintenance of species boundaries. *Molecular Ecology* **23**: 1668–1678.

Levin, D.A. 1975. Interspecific hybridization, heterozygosity, and gene exchange in *Phlox*. *Evolution* **29**: 37-51.

Levin, D.A. 1983. Polyploidy and novelty in flowering plants. *The American Naturalist* **122**: 1-25.

Levin, D.A. 2000. *The Origin, Expansion, and Demise of Plant Species*. Oxford University Press, USA. New York.

Linder, H.P. 2003. The radiation of the Cape flora, southern Africa. *Biological Reviews* **78**: 597-638.

Lijtmaer, D.A., Mahler, B., and Tubaro, P.L. 2003. Hybridization and Postzygotic Isolation Patterns in Doves and Pigeons. *Evolution* **57**: 1411-1418.

Losos, J.B, and Ricklefs, R.E. 2009. Adaptation and diversification on islands. *Nature* **457**: 830-836.

Lowry, D.B., Modliszewski, J.L., Wright, K.M., Wu, C.A., and Willis, J.H. 2008. The strength and genetic basis of reproductive isolating barriers in flowering plants. *Philosophical Transactions of the Royal Society B: Biological Sciences* **363**: 3009-3021.

Lowry, D.B. 2012. Ecotypes and the controversy over stages in the formation of new species. *Biological Journal of the Linnean Society* **106**: 241-257.

Lu, J., Peatman, E., Tang, H., Lewis, J., and Liu, Z. 2012. Profiling of gene duplication patterns of sequenced teleost genomes: evidence for rapid lineage-specific genome expansion mediated by recent tandem duplications. *BMC Genomics* **13**: 2-10.

Ludwig, S., Robertson, A., Rich, T.G.C., Djordjević, Cerović, Houston, L., Harris, S.A., and Hiscock, S.J. 2013. Breeding systems, hybridization and continuing evolution in Avon Gorge *Sorbus* **111**: 563-575.

Mable, B.K. 2004. Why polyploidy is rarer in animals than in plants: myths and mechanisms. *Biological Journal of the Linnean Society* **82**: 453-466.

Mallet, J. 2005. Hybridization as an invasion of the genome. *Trends in Ecology & Evolution, Special issue: Invasions, guest edited by Michael E. Hochberg and Nicholas J. Gotelli* **20**: 229-237.

Mallet, J. 2007. Hybrid speciation. *Nature* **446**: 279-283.

Martin, N.H., and Willis, J.H. 2007. Ecological Divergence Associated with Mating System Causes Nearly Complete Reproductive Isolation Between Sympatric *Mimulus* Species. *Evolution* **61**: 68-82.

Manning, H.P., and Linder, J.C. 1992. Pollinators and evolution in *Disperis* (Orchidaceae), or why are there so many species? *South African Journal of Science* **88**: 38-49.

Martin, N.H., and Willis, J.H. 2010. Geographical variation in postzygotic isolation and its genetic basis within and between two *Mimulus* species. *Proceedings of the Royal Society B: Biological Sciences* **365**: 2469-2478.

Martinsen, G.D., Whitman, T.G., Turek, R.J., and Keim, P. 2001. Hybrid populations selectively filtrt gene introgression between species. *Evolution* **55**: 1235-1335.

Masterson, J. 1994. Stomatal Size in Fossil Plants: Evidence for Polyploidy in Majority of Angiosperms. *Science* **264**: 421-424.

Mayr, E. 1942. *Systematics and the Origin of Species, from the viewpoint of a Zoologist*. Columbia University Press. New York. M.

Mavárez, J, and Linares, M. 2006. Homoploid hybrid speciation in animals. *Molecular Ecology* **17**: 4181-4185.

Mazer, S.J., Dudley, L.S., Hove, A.A., Emms, S.K., and Verhoeven, A.S. 2010. Physiological performance in *Clarkia* sister taxa with contrasting mating systems: do early-flowering autogamous taxa avoid water stress relative to their pollinator-dependent counterparts? *International Journal of Plant Sciences* **171**: 1029-1047.

McCall, C., and Primack, R.B. 1992. Influence of flower characterisitics, weather, time of day and season on insect visitation rates in three plant communities. *American Journal of Botany* **79**: 434-442.

McGrath, C., and Lynch, M. 2012. Evolutionary significance of whole-genome duplication in *Polyploidy and genome evolution*.1-20. Springer.

Michener, C.D. 1970. Diverse approaches to systematics. *Evolutionary Biology* **4**: 1-38.

Mishler, B.D., and Donoghue, M.J. 1982. Species concepts: a case for pluralism. *Systemaic Zoology* **31**: 491-503.

Melo, M.C., Grealy, A., Brittain, B., Walter, G.M., and Ortiz-Barrientos, A. 2014. Strong intrinsic reproductive isolation between parapatric populations of an Australian groundsel. *New Phytologist* **203**: 323-334.

Meudt, H.M., and Clarke, A.C. 2007. Almost forgotten or latest practice? AFLP applications, analyses and advances. *Trends in Plant Science* **12**: 106-117.

Morin, R.D., Petrscu, A., Liao, N., Griffith, M., Chow, W., Kirkpatrick, R., Butterfield, S.Y., Young, A.C., Stott, J., Barber, S., Babakaiff, R., Dickson, M.C., Matsuo, C., Wong, D., Yang, G.S., Smailus, D.E., Wetherby, K.D., Kwong, P.N., Grimwood, J., Brinkley 3rd, C.P., Brown-John, M., Reddix-Dugue, N.D., Mayo, M., Schmutz, J., Beland, J., Park, M., Gibson, S., Olson, T., Bouffard, G.G., Tsai, M., Featherstone, R., Chand, S., Siddiqui, A.S., Jang, W., Lee, E., Klein, S.L., Blakesley, R.W., Zeeberg, B.R., Narasimhan, S., Weinstein, J.N., Pennacchio, C.P., Wagner, L., Gerhard, D.S., Marra, M.A., Jones, S.J.M., and Holt. R.A. 2006. Sequencing and analysis of 10, 967 full-length cDNA clones from *Xenopus laevis* and *Xenopus tropicalis* reveals post-tetraploidization transcriptome remodeling. *Genome Research* **16**: 796-803.

Moyle, L.C., Olson, M.S., and Tiffin, P. 2004. Patterns of reproductive isolation in three angiosperm genera. *Evolution* **58**: 1195-1208.

Mueller, U. G and Wolfenbarger, L. L. 1999. AFLP genotyping and fingerprinting. *Trends in Ecology & Evolution* **14**: 389-394.

Muller, H.J. 1940. Bearing of the *Drosophila* work on systematics. In: Huxley J, editor. *The new systematics*. Clarendon Press. Oxford. 185-268.

Nanda, R., Bhargava, S.C., Tomar, D.P.S., and Rawson, H.M. 1996. Phenological development of *Brassica campestris, B. juncea, B. napus* and *B. carinata* grown in controlled environments and from 14 sowing dates in the field. *Field crops research* **46**: 93-103.

Nixon, K.C and Wheeler, Q.D. 1990. An amplification of the Phylogenetic Species Concept. *Cladistics* **6**: 211-223.

Ollerton, J and Lack, A.J. 1992. Flowering phenology: An example of relaxation of natural selection? *Trends in Ecology & Ecology* **7**: 274-276.

Orr, H. A and Presgraves, D. C. 2000. Speciation by postzygotic isolation: forces, genes and molecules. *BioEssays* **22**: 1085-1094.

Orr, H. A and Turelli, M. 2001. The evolution of postzygotic isolation: accumulating Dobzhansky-Muller incompatibilities. *Evolution* **55**: 1085-1094.

Otto, F. 1992. Preparation and staining of cells for high-resolution DNA analysis. In: Radbruch, ed. *Flow cytometry and cell sorting*. Springer-Verlag, Berlin. 101-104.

Otto, S.P., and Whitton, L. Polyploid incidence and evolution. 2000. *Annual Review of Genetics* **34**: 401-437.

Peakall, R, and Smouse, P.E. 2012. GenAlEx 6.5: genetic analysis in Excel. Population genetic software for teaching and research- an update. *Bioinformatics* **28**: 2537-2539.

Pickup, M., Barton, N.H., Brandvain, Y., Fraisse, C., Yakimowski, S., Dixit, T., Cereghetti, E., and Field, D. 2019. Mating system variation in hybrid zones: Facilitation, barriers and asymmetries to gene flow. *New Phytolgist* **224**: 1035-1047.

Poorter, H., Fiorani, F., Pieruschka, R., Wojciechowski, T., van der Putten, W.H., Kleyer, M., Schurr, U., and Postma, J. 2016. Pampered inside, pestered outside? Differences and similarities between plants growing in controlled conditions and in the fiels. *New Phytologist* **212**: 838-855.

Postlethwaite, J.H., Woods, I.G., Ngo-Hazelett, Yan, P., Kelly, Y.L., Chu, F., Huang, H., Hillforce, A., and Talbot, W.S. 2000. Zebrafish Comparative Genomics and the Origins of Vertebrate Chromosomes. *Genome research* **10**: 1890-1902.

Presgraves, D. C. 2002. Patterns of postzygotic isolation in Lepidoptera. *Evolution* **56**: 1168-1183.

Pritchard, J.K., Stephens, M., and Donelly, P. 2000. Inference of population structure using multilocus genotype data. *Genetics* **155**: 945-959.

Ramsey, J., and Schemske, D.W. 1998. Pathways, Mechanisms, and Rates of Polyploid Formation in Flowering Plants. *Annual Review of Ecology and Systematics* **29**: 467-501.

Ramsey, J., and Schemske, D.W. 2002. Neopolyploidy in Flowering Plants. *Annual Review of Ecology and Systematics* **33**: 589-639.

Ramsey, J., Bradshaw, H.D., Schemske, D.W. 2003. Components of reproductive isolation between the monkeyflowers *Mimulus lewisii* and *M. cardinalis* (Phrymaceae). *Evolution* **57**: 1520-1534.

Rieseberg, L.H. 1991. Homoploid Reticulate Evolution in *Helianthus* (Asteraceae): Evidence from Ribosomal Genes. *American Journal of Botany* **78**: 1218-1237.

Rieseberg, L.H., Ellstrand, N.C, and Arnold, M. 1993. What Can Molecular and Morphological Markers Tell Us About Plant Hybridization? *Critical Reviews in Plant Sciences* **12**: 213-241.

Rieseberg, L.H. 1995. The Role of Hybridization in Evolution: Old Wine in New Skins. *American Journal of Botany* **82**: 944-953.

Rieseberg, L.H. 1997. Hybrid Origins of Plant Species. *Annual Review of Ecology and Systematics* **28**: 359-389.

Rieseberg, L.H., Raymond, O., Rosenthal, D.M., Lai, Z., Livingstone, K., Nakazato, T., Durphy, J.L., Schwarzbach, A.E., Donovan, L.A., Lexer, C. 2003. Major ecological transitions in wild sunflowers facilitated by hybridization. *Science* **301**: 1211-1216.

Rieseberg, L.H, and Willis, J.H. 2007. Plant Speciation. *Science* **317**: 910-914.

Robertson, K., Goldberg, E.E., and Ilgić. 2010. Comparative evidence for the correlated evolution of polyploidy and self-compatibility in Solanaceae. *Evolution* **65**: 139-155.

Rogers, S.M and Bernatchez, L. 2006. The genetic basis of intrinsic and extrinsic post-zygotic reproductive isolation jointly promoting speciation in the lake whitefish species complex (*Coregonus clupeaformis*). *Journal of Evolutionary Biology* **19**: 1979-1994.

Rosenberg, N.A., Pritchard, J.K., Weber, J.L., Cann, H., Kidd, K.K., Zhivotovsky, L.A., and Feldman, M.W. 2002. Genetic structure of human populations **298**: 2381-2385.

Rundle, H. D., and Nosil, P. 2005. Ecological speciation. *Ecology Letters* **8**: 336-352.

Runquist, B.R.D., Chu, E., Iverson, J.L., Kopp, J.C., and Moeller D.A. 2014. Rapid evolution of reproductive isolation between incipient outcrossing and selfing *Clarkia* species. *Evolution* **68**: 2885-2900.

Scopece, G., Widmer, A., and Cozzolino, S. 2008. Evolution of postzygotic reproductive isolation in a guild of deceptive orchids. *The American Naturalist* **171**:315-326.

Scott, R.J., Spielman, M., Bailey, J., and Dickinson, H.G. 1998. Parent-of-origin effects on seed development in *Arabidopsis thaliana. Development* **125**: 3329-3341.

Schemske, D. W. 2000. Understanding the origin of species. *Evolution* **54**: 1069-1073.

Schemske, D. W and Bradshaw, H. D. 1999. Pollinator preference and the evolution of floral traits in monkeyflowers (*Mimulus*). *Proceedings of the National Academy of Sciences* **96**:11910-11915.

Schatlowski, N., and Köhler, C. 2012. Tearing down barriers: understanding the molecular mechanisms of interploidy hybridizations. *Journal of Experimental Biology* **63**: 6059-6067.

Schluter, D. 1996. Adaptive radiation along genetic lines of least resistance. *Evolution* **50**: 1766-1774.

Schluter, D. 2000. Ecological character displacement in adaptive radiation. *The American Naturalist* **156**: 4-16.

Schluter, D. 2009. Evidence for ecological speciation and its alternative. *Science* **323**: 737-741.

Schumer, M., Rosenthal, G.G., and Andolfatto, P. 2014. How common is homoploid hybrid speciation? *Evolution* **68**: 1553-1560.

Scopece, G., Widmer, A., and Cozzolino, S. 2008. Evolution of postzygotic reproductive isolation in a guild of deceptive orchids. *The American Naturalist* **171**: 315-326.

Simpson, G.G., 1961. *Principles of Animal Taxonomy*. Colombia University Press. New York.

Sites, J.W., and Marshall, J. 2003. Delimiting species: A Renaissance issue in systematic biology. *Trends in Ecology and Evolution* **18**: 462-470.

Skrede, I., Borgen, L., and Brochmann, C. 2009. Genetic structuring in three closely related circumpolar plant species: AFLP versus microsatellite markers and high-arctic versus arctic-alpine distributions. *Heredity* **102**: 293-302.

Slotte, T., Huang, H., Lascoux, M., and Ceplitis, A. 2008. Polyploid Speciation Did Not Confer Instant Reproductive Isolation in *Capsella* (Brassicaceae). *Molecular Biology and Evolution* **25**: 1472-1481.

Smith, J.M. 1966. Sympatric Speciation. *The American Naturalist* **100**: 637-650.

Sobel, J.M., Chen, G.F., Watt, L.R., Schemske, D.W. 2010. The Biology of Speciation. *Evolution* **64**: 295-315.

Sonnleitner, M., Weis, B., Flatscher, R., Garcia, P.E., Suda, J., Krejčíková, Schneeweiss, G.M., Winkler, M., and Schönswetter, P. 2013. *PLoS One* **8**: e78959.

Stebbins, G.L. 1950. *Variation and evolution in plants*. Oxford University Press, London.

Stebbins, G.L. 1957. Regularities of transformation in the flower. *Evolution* **11**: 106-108.

Stebbins, G.L. 1971. *Chromosomal evolution in higher plants*. Oxford University Press, London.

Suni, S.S and Hopkins, R. 2018. The relationship between postmating reproductive isolation and reinforcement in *Phlox*. *Evolution* **72**: 1387-1398.

Sutherland, B.L. and Galloway, L.F. 2017. Postzygotic isolation varies by ploidy level within a polyploidy complex. *New Phytologist* **213**: 401-412.

Sutherland, B.L., Quarles, B.M., and Galloway, L.F. 2018. Intercontinental dispersal and whole-genome duplication contribute to loss of self-incompatibility in a polyploid complex. *American Journal of Botany* **105**: 249-256.

Sweigart, A.L., Mason, A.R., and Willis, J.H. 2007. Natural variation for a hybrid incompatibility between two species of *Mimulus*. *Evolution* **61**: 141-151.

Taylor, E.B., Boughman, J.W., Groenenboom, M., Sniatynski, M., Schluter, D., and Gow, J.L. 2006. Speciation in reverse: morphological and genetic evidence of the collapse of a three-spined stickleback (*Gasterosteus aculeatus*) species pair. *Molecular Ecology* **15**: 343-355.

Tiffin, P., Olson, S.M., and Moyle, L.C. 2001. Asymmetrical crossing barriers in angiosperms. *Proceedings of the Royal Society B: Biological Sciences* **268**: 861-867.

Turelli, M., and Moyle, L.C. 2007. Asymmetric Postmating Isolation: Darwin's Corollary to Haldane's Rule. *Genetics* **176**: 1059-1088.

Turelli, M., and Orr, H.A. 2000. Dominance, epistasis, and the genetics of postzygotic isolation. *Genetics* **154**: 1663-1679.

Tyagi, A., Singh, S., Mishra, P., Singh, A., Tripathi, A.M., Jena, S.N., and Roy, S. 2016. Genetic diversity and population structure of *Arabidopsis thaliana* along an altitudinal gradient. *Annals of Botany Plants* **8**: 1-17.

van der Niet, T., Hansen, D.M., and Johnson, S.D. 2011. Carrion mimicry in a South African orchid: flowers attract a narrow subset of the fly assemblage on animal carcasses. *Annals of Botany* **107**: 981-992.

Vos, P., Hogers, R., Bleeker, M., Reijans, M., Van de Lee, T., Hornes, M., Frijters, A., Pot, J., Peleman, J., and Kuiper, M. 1995. AFLP: a new technique for DNA fingerprinting. *Nucleic Acids Research* **23**: 4407-4414.

Wendel, J., and Doyle, J. 2005. Polyploidy and evolution in plants. In: Henry, R.J. *Plant diversity and evolution: genotypic and phenotypic variation in higher plants.* Wallingford. 97-117.

Whitehead, M.R., and Peakall, R. 2014. Pollinator Specificity Drives Strong Prepollination Reproductive Isolation in Sympatric Sexually Deceptive Orchids. *Evolution* **68**: 1561-1575.

Wiens, J.J. 2007. Species Delimitation: New Approaches for Discovering Diversity. *Systematic Biology* **56**: 875-878.

Widmer, A., Lexer, C., and Cozzolino, S. 2009. Evolution of reproductive isolation in plants. *Heredity* **102**: 31-38.

Wiley, E.O. 1981. *Phylogenetics: The Theory and Practice of Phylogenetic Systematics*. Wiley-Blackwell. New York.

Wiley, C., Qvanström. A., Andersson, G., Borge, T., and Sætre G. 2009. Postzygotic Isolation Over Multiple Generations of Hybrid Descendants in A Natural Hybrid Zone: How Well Do Single-Generation Estimates Reflect Reproductive Isolation? *Evolution* **63**: 1731-1738.

Wolf, J.B., and Wade, M.J. 2009. What are maternal effects (and what are they not)? *Philosphical Transactions of the Royal Society London B*: *Biological Sciences* **364**: 1107-1115.

Wolfe, K.H., and Shields, D.C. 1997. Molecular evidence for an ancient duplication of the entire yeast genome. *Nature* **387**: 709-712.

Wood, T.E., Takebayashi, N., Barker, M.S., Mayrose, I., Greenspoon, P.B., and Rieseberg, L.H. 2009. The frequency of polyploidy speciation in vascular plants. *Proceedings of the National Academy of Sciences* **106**: 13875-13879.

Wu, C.I., and Beckenbach, A.T. 1983. Evidence for extensive genetic differentiation between the sex-ratio and the standard arrangement of *Drosophila pseudoobscura* and *D. persimilis*. *Genetics* **105**: 71-86.

Yang, C.Y., Gituru, R.W, and Guo, Y. 2007. Reproductive isolation of two sympatric louseworts, *Pedicularis rhinathoides* and *Pedicularis longiflora* (Orobanchaceae): how does the same pollinator type avoid interspecific pollen transfer? *Biological Journal of the Linnean Society* **90**: 37-48.

Yatabe, Y., Kane, Scotti-Saintagne, C., and Rieseberg, L.H. 2007. Rampant gene exchange across a strong reproductive barrier between the annual sinflowers, *Helianthus annuus* and *H. petiolaris. Genetics* **175**: 1883-1893.

Yi, L and Maki, M. 2015. Variation in the frequency and extent of hybridization between *Leucosceptrum japonicum* and *L. stellipilum* (Lamiaceae) in the Central Japanese Mainland. *Public Library of Science One* **10**: e0116411.

Yost, J.M and Kay, K.M. 2009. The evolution of postpollination reproductive isolation in *Costus*. *Sexual Plant Reproduction* **22**: 247-255.