Investigating hybridization among high altitude *Rhodohypoxis* species in the Drakensberg Alpine Centre

by

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DECLARATION

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

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ABSTRACT

Putative hybridization between the alpine *Rhodohypoxis* taxa (*R. baurii* var. *confecta*, *R. deflexa*, *R. rubella*, *R. incompta*, and *R. thodiana*) as well as *Hypoxis parvula* var. *parvula* has been documented in the wild – suggesting that there are few genetic barriers between these taxa that often grow in sympatry. This study aimed to assess patterns of hybridization among the alpine *Rhodohypoxis* species and between them and *H. parvula* var. *parvula* at three different natural study sites and to contribute new data that might help delineate/solidify genus and species boundaries in this group using morphometric analysis, seed surface micromorphological comparisons, microsatellite data, and controlled greenhouse crosses. It was found that although all the methodologies were congruent in their ability to delineate the *Rhodohypoxis* species from one another and from *H. parvula* var. *parvula* (thereby corroborating their current circumscription), substantial admixture was detected at each of the three study sites – and admixed individuals were largely morphologically cryptic. Furthermore, morphometric analysis and seed surface micromorphology could not distinguish between *R. thodiana* and putative hybrid taxa – suggesting it may be of hybrid origin. The present study also reports the complete self-incompatibility of polyploid *H. parvula* var. *parvula*, the facultative self-compatibility of diploid *R. baurii* var. *confecta*, the successful crossbreeding of these two taxa from Sentinel Peak with a strong asymmetrical barrier to gene exchange, and a mechanism involving double-reduced polyploid pollen facilitating this interploidy cross. Low germination rate of the resulting hybrid seed is consistent with a decrease in F1 viability. These findings, coupled with the low incidence of polyploid *H. parvula* var. *parvula* individuals producing double reduced pollen, and the low levels of admixture detected, suggesting substantial post-pollination reproductive isolation between these two taxa. Nevertheless, caution should be taken when making strong taxonomic inferences from phylogenies that include *R. baurii* var. *confecta* and *H. parvula* var. *parvula* representatives from the Sentinel Peak study site.

CHAPTER 1: INTRODUCTION

GENERAL INTRODUCTION

Evolution, the descent with modification or change in the form and physiology of organisms over many generations, is the unifying principle in biology. Evolutionary change was originally thought to take place in a diverging tree-like pattern of lineages first illustrated by Darwin (1859), but modern genetics revealed that this view is somewhat misleading. In actuality, the process is more likely represented as an 'impenetrable thicket' or web-like – largely due to hybridization (Sample, 2009). Plant and animal species readily crossbreed to produce potentially viable offspring. According to some estimates, between one and ten percent of animals and ~25% of vascular plants regularly form interspecific hybrids (Grant & Grant, 1992; Mallet, 2005; Schwenk, 2008).

Hybridization

Hybridization is defined as the mating of members of genetically diverse individuals of different species and has been considered 'evolutionary noise' by some (i.e., inconsequential to the main process of evolution; Wagner et al., 1970; Futuyma & Antonovics, 1992; Mayrose et al., 2011). Rare hybridization between two strongly allopatric species may have little to no long-term impact on the gene pool of either species, especially if the hybrids are sterile. This scenario, which is presumably rather common, potentially reflects evolutionary noise. In contrast, hybridization, especially when combined with polyploidy, is thought to lead to speciation and the development of new phenotypes that fill new niches by others (Soltis & Soltis, 2003; Rieseberg et al., 2003; Soltis & Soltis, 2009). The study of hybridization in plants can be divided into two categories, 1) hybridization frequency and 2) evolutionary consequences of hybridization (Grant, 1981). Hybrid zones provide a unique insight into both these processes (Ekrt et al., 2010). Hybrid zones are small areas where genetically diverse populations come into contact, mate, and produce hybrid offspring (Barton & Hewitt, 1985). In order to study speciation, the nature of species, and species limits, hybrid zones can be used as natural laboratories (Barton & Hewitt, 1989; Harrison, 1990).

Some researchers contend that in order to produce genetic novelty within populations, hybridization is more significant than mutation (Stebbins, 1959; Knobloch, 1972; Gross et al.,

2004). The advancements in genome-based research, the increased availability of relevant techniques, and studies on a few model plant systems (e.g., *Tragopogon* L.; Soltis et al., 2004) demonstrate how gene flow between divergent taxa can lead to new phenotypic variation, enable adaptation to novel environments, and support speciation (Arnold et al., 2004; Schmickl & Koch, 2011). Although large-scale phylogenetic patterns of hybridization are poorly understood, they play a major role in the evolution of many taxonomic plant groups. This knowledge gap has contributed to ongoing interest in plant hybridization and introgression (Whitney et al., 2010).

Understanding which plant families and species are prone to hybridization is critically important because other organisms not involved in the hybridization process may suffer significant consequences. For instance, hybridization has been discovered to be a key factor in the invasive success of polyploid *Spartina* Schreb. species (Ainouche et al., 2009). While spontaneous natural hybridization occurs more frequently in some plant families and genera than others, it is most prevalent in outcrossing species that have reproductive strategies that stabilise hybridity, such as vegetative reproduction, persistent odd polyploidy, or agamospermy (Ellstrand et al., 1996). These mechanisms often allow for persistence in the face of challenges such as the minority cytotype exclusion principle (MCE) which, in turn, may result in the establishment of new and distinct evolutionary lineages over time (Mallet, 2005).

From a taxonomic perspective, it has long been recognized that major plant lineages hybridise (Grant, 1981). Taxonomy is the science that explores, describes, names, and classifies all organisms (Rouhan & Gaudeul, 2014). Natural hybridization obscures species boundaries and alters variability in natural populations, which has considerable implications for taxonomy. For instance, the Linnaean classification system does not make provision for organisms that are in the very early process of speciation – organisms that are in this transitional state may be lumped in with their close relatives (Green, 1996). It may be possible to accommodate a F1 hybrid within the Linnaean system with an unambiguous designation such as "*Clivia nobilis* Lindl. × *C. gardenii* Hook. With the × indicating that the taxon is a hybrid between the two mentioned species. Even though most natural hybrids are transient and only a small fraction of them survive to establish stable nothospecies (defined as a hybrid which is formed by direct hybridization of two species, not other hybrids), it became

standard among botanists to officially describe all newly discovered hybrids as nothospecies according to published etiquette (Harrison, 1990; 1993).

Recently, for species groups that are subject to frequent hybridization (and polyploidization), application of the general lineage concept (GLSC; De Queiroz 2007; Padilla-García et al., 2018; Niemann et al., 2013) has been suggested to facilitate appropriate species delineation. The GLSC defines a species as a group of independently evolving metapopulation lineages distinguished by various traits acquired by the species throughout the process of speciation (such as reproductive isolation, morphological variations, or genetic differences. The GLSC has garnered broad acceptance and sparked the development of an integrative taxonomic approach that uses a variety of complementary methods in tandem to draw boundaries between species (Dayrat, 2005). This approach argues that the basis for taxonomic inference should be congruence across different types of characters and analyses. When data from different sources are incongruent, it is preferable to refrain from delimiting species (Carstens et al., 2013).

Plant morphology refers to the plant's physical form and structure of the plant. Traits can be used to visually identify plants which allows the great diversity of these organisms to be identified and classified. Plant morphology not only characterises the shape and structure of the plant, but also the role of each part, allowing researchers to pinpoint the origin and composition of the plant (Evert et al., 2012). Because they are simple to observe and useful for use in keys and descriptions, the features of floral morphology are frequently employed as the most important characters in flowering plant classification (De Craene & Louis, 2022).Given that many putative hybrids that were detected solely based on morphology have subsequently been verified as hybrids using molecular methods, morphological features remain a potentially valuable indicator of plant hybridization (Small et al., 2004).

Plant evolution has shaped significant morphological and developmental innovations that have improved the success of plants as terrestrial organisms (Mora & González, 2016). The variation observed in plant traits may be ascribed to genetic and environmental reasons. Even though plant populations are phenotypically diverse, most of the evolution-orientated, trait-based research has been directed towards species differences. The general understanding that plant trait variation between species is often greater than differences within species, justifies this widely adopted approach (Umaña & Swenson, 2019). Removing plants from their natural habitat and cultivating them artificially can yield valuable information regarding

plant trait variation. For example, when the phenological development of *Brassica* L. species in a controlled environment was compared to their phenological development in their natural environment, it was discovered that the plants in the controlled environment flowered earlier than those in their natural environment – showing that flowering time is an environmentally variable character (Nanda et al., 1996).

While morphological intermediacy is a distinguishing feature used to identify hybrids in the field, individuals with hybrid backgrounds may exhibit novel features/traits, or they may be anatomically cryptic – closely resembling their parents yet having mixed genetic histories (Dittrich-Reed & Fitzpatrick, 2013). There has been a recent surge in the interest in plant hybridization and introgression because of the development of next-generation sequencing technology and the availability of genomic data sets. These datasets include the creation of tools like microsatellites to identify gene flow and to track alleles moving between lineages (Payseur & Rieseberg, 2016; Abbott et al., 2013). Microsatellites are tandem repetitive sequences with repeating units containing one to six nucleotides, commonly known as short tandem repeats (STRs) or simple sequence repeats (SSRs). Since microsatellites are susceptible to slippage during DNA replication, the number of repetitions is highly varied both within populations and within an individual's alleles (Kalia et al., 2011). Different alleles at a locus are distinguished by various repeat unit counts. The same information is provided by them as by allozymes: distinct loci with codominant alleles, but they are more consistently neutral and have a higher rate of variability. Microsatellite alleles are often inherited in a Mendelian manner, similar to allozymes (O'Connell & Wright, 1997).

The affordability and utility of microsatellites in large-scale genotyping makes them ideal markers for population genetics studies. In fingerprinting applications including cultivar and variety discrimination, marker-assisted breeding, map-based cloning, and gene flow studies, microsatellite-based markers are dependable and straightforward to use (Kalia et. al., 2011). Microsatellites can also be used to evaluate hybridization, where their codominant nature is crucial and enables the identification of each parent's allelic contribution in both sexual and somatic hybrids (Abdul-Muneer, 2014; Turchetto et al., 2015). Due to their hypervariable character and extensive genome coverage, microsatellites have become a preferred marker for a variety of applications in plants. The additional appeal of PCR-based approaches, which only require minimal amounts of DNA, has resulted in widespread use of this method (Abdul-Muneer, 2014; Turchetto et al., 2015).

Polyploidy

Polyploidy is a phenomenon where organisms have multiple sets of chromosomes instead of the common two sets in the sporophyte plant body (Soltis & Soltis, 2016). Plants are particularly known for being able to undergo polyploidization events, resulting in increased genome size. It is estimated that up to ~70% of all plant species have undergone polyploidization at some point in their evolutionary history (Grant, 1981; Masterson, 1994; Leitch & Leitch, 2008; Wood et al. 2009). Although it has been deemed as an evolutionary dead end by some (Stebbins, 1950; Stebbins, 1971; Mayrose at al., 2011; Arrigo & Barker, 2012; Mayrose et al., 2015), it is argued by others to have contributed to the success and diversification of plants, allowing them to adapt to changing environments and develop new traits by having extra copies of genes freed up to undergo neofunctionalization (Otto & Whitton, 2000; Adams & Wendel, 2005; Soltis et al., 2009a; Madlung, 2013; Soltis et al., 2014a; Soltis et al., 2014b). Polyploid plants have been the subject of much research in fields such as genetics, evolution, and agriculture. Studying the genetic changes that occur during polyploidization can provide insights into the mechanisms of speciation and adaptation (Soltis & Soltis, 2016).

Polyploidy can occur naturally, such as through hybridization events (allopolyploidy) or errors in cell division such as nondisjunction (autopolyploidy). Gametes are the reproductive cells produced by plants and animals, and they play a crucial role in sexual reproduction. In plants, the male gametes are called pollen grains, which are produced in the anthers of the flower. Each pollen grain contains two haploid cells: a generative cell and a tube cell (Nagaharu & Nagaharu, 1935). The generative cell will later divide into two sperm cells, while the tube cell grows into a pollen tube that delivers the sperm cells to the female gametes, located in the ovary of the flower. Before fertilisation can occur, the microspore mother cells within the anthers and the megaspore mother cells within the ovule must undergo a process called meiosis, which reduces the number of chromosomes in each cell by half. This process ensures that when the sperm cells from the mature pollen combine with the mature female gametes, the resulting zygotes will have the correct number of chromosomes for the species (Birky, 1993). However, in some cases, meiosis may not occur properly, resulting in unreduced gametes or the production of a mono haploid gamete. Research on agricultural systems has shown that gametic abnormality may cause recurrent polyploid development in plant populations (Harlan & de Wet, 1975; Bretagnolle & Thompson, 1995). Chromosome numbers can also be altered through the elimination of chromosomes in early

cell division following fertilisation and result in extraordinary cross-hybridization opportunities (Dweikat, 2005; Cox, 2018).

Polyploid plants often display alterations in various traits, such as pollen size, vegetative plant body, floral structures and stomatal size (Lutz, 1907; Maherali et al., 2009; Balao et al., 2011; Porturas et al., 2019). In diploid plants, pollen grains typically have a single chromosome copy (denoted by X). However, the influence of polyploidy can elevate the number of chromosome copies in pollen grains from X to 2X or beyond. This increase in DNA content theoretically leads to an enlargement in the size of pollen grains (Dufaÿ et al., 2014). It has thus been argued that polyploid plants generate larger pollen grains than their diploid counterparts (Porturas et al., 2019). In principle, the effect of polyploidy should result in an increased size for nearly all pollen grains, especially in polyploids with even-numbered chromosomal levels (Lutz, 1907; Segraves, 2017 Porturas et al., 2019; Clo & Kolář, 2021). Overall, there is certain evidence indicating a potential association between ploidy level and pollen size, although exceptions have been reported (Porturas et al., 2019; Becker et al. 2022). For example, the genus *Oxalis* L. is notable for its widespread polyploidy, but researchers discovered no consistent differences in pollen size between diploid and polyploid individuals (Becker et al., 2022).

Reinforcement

Reinforcement takes place when natural selection favours characteristics that reduce hybridization, as hybrids tend to be sterile, inviable, or poorly adapted (Dobzhansky, 1937). Numerous studies spanning various taxonomic groups have identified indications of reinforcement, suggesting that the process can significantly contribute to speciation (Servedio & Noor, 2003; Hopkins ,2013). A good example of this is strong reinforcing selection for increased pigment intensity in *Phlox drummondii* Hook. in areas where it co-occurs with the closely related species *Phlox cuspidata* Scheele – a species with which it can hybridize (Hopkins & Rausher, 2012; Hopkins, 2013). This increased pigment intensity in *P. drummondii*, which is not seen in populations where *P. cuspidata* is absent, results in reduced movement of pollinators between *P. drummondii* and *P. cuspidata* (Hopkins & Rausher, 2012).

Elevated costs related to hybridization are often seen across different taxonomic groups and can arise from various post-mating reproductive isolation (RI) barriers, including gametic incompatibilities, hybrid inviability and/or hybrid sterility (Coyne & Orr, 2004).

Examining the strength and relative significance of these barriers in contributing to overall RI can provide insights into the primary factors motivating selection against hybridization that drives the reinforcement process (Ramsey et al., 2003; Kay, 2006; Baack et al., 2015; Suni et al., 2020). The time since the divergence of two lineages also might impact the potency of post-mating reproductive isolation (Turissini et al., 2018). A positive correlation between the strength of postzygotic RI and phylogenetic divergence is expected, as lineages that separated a long time ago would have had more opportunities to accumulate and establish mutations that contribute to reproductive isolation (Mummery-Widmer et al., 2009).

STUDY SYSTEM

Hypoxidaceae R. Br, which belongs to the order Asparagales, encompasses approximately 8 genera and ca. 160 species with a global distribution (Birch & Kocyan, 2021). The issue of generic limits within the Hypoxidaceae family has always posed a challenging question, reflected by the different circumscriptions applied to the family (Burtt, 2000). In the phylogenetic study of Kocyan et al. (2011), it was found that *R. baurii* var. *confecta* and *H. parvula* var. *parvula* grouped together – rendering both *Hypoxis* and *Rhodohypoxis* non-monophyletic. These findings led to the suggestion that *Rhodohypoxis* and *Hypoxis* should be unified into a single genus.

Polyploidy and hybridization greatly exacerbate the difficulty of phylogenetic reconstruction (Grant, 1981; Mallet, 2001; Rieseberg et al., 2003; Padilla-García et al., 2018). Past hybridization can lead to shared plastid haplotypes among species (Palme et al., 2004) and this could also explain why these two taxa grouped together. Although phylogenetic analyses are an effective method for identifying relationships within families, they are unable to address the issue of what taxonomic rank should be assigned to the resulting monophyletic groups.

In the current study *Hypoxis* L. and *Rhodohypoxis* Nel are treated as distinct genera since the outstanding requirement for well-supported re-circumscription is a full phylogenetic analysis of all the species of Hypoxidaceae present in South Africa. Until such an analysis is conducted, any implementation of changes to the circumscription of *Rhodohypoxis* is deemed premature (Niemann et al., 2023).

Figure 1. Images of floral features of **A)** *Hypoxis* and **B)** *Rhodohypoxis*. The stigma, style, and the stamens are exposed in *Hypoxis* but are hidden by tepals in *Rhodohypoxis*.

***Rhodohypoxis* Nel**

Rhodohypoxis is a small genus of six species demarcated in 1914 by G. Nel, who distinguished it from *Hypoxis* because of its closed pink or white flowers. Hilliard & Burtt (1978) as well as Duncan (2003) compiled comprehensive descriptions of the genus. *Rhodohypoxis* individuals are small perennial geophytes that die back in the cold winter months. Their distribution is centred in the Drakensberg, but some isolated populations are known in Mpumalanga, Limpopo, and Eswatini (Hilliard & Burtt, 1978).

To date, there is limited information on pollinator visitation for *Rhodohypoxis* flowers, but a few generalist pollinators have been observed (Ferreira et al., in prep). In this study, we will focus on the five alpine *Rhodohypoxis* species that occur between 2500 m.a.s.l. to 3500 m.a.s.l. since they often co-occur in sympatry and hybridization has been reported between some of them (Hilliard & Burtt, 1978). Below is a summary of relevant information on each taxon as it pertains to this study:

Rhodohypoxis baurii (Baker) Nel var. *confecta* Hilliard & B.L.Burtt

Description: *R. baurii* var. *confecta* has bright green and slightly hairy leaves. The flowers are usually pale to bright pink or white.

Distinguishing characters: *Rhodohypoxis baurii* var. *confecta* is distinguished from the other two *R. baurii* varieties by displaying different coloured flowers on the same plant (*Rhodohypoxis baurii* (Baker) Nel var. *baurii* has predominantly dark pink flowers, while *Rhodohypoxis baurii* (Baker) Nel var. *platypetala* (Baker) Nel has predominantly white flowers).

Habitat and distribution: This taxon occurs in KwaZulu-Natal, Eastern Cape, the Free State, Limpopo, Lesotho and Swaziland. It is found growing on damp grassy slopes and on rock sheets from 1900 m.a.s.l. to 2900 m.a.s.l. It is the *R. baurii* variety, that occurs at the highest altitudes, and the only variety that therefore grows in sympatry with the other four alpine *Rhodohypoxis* species. Flowers from October to February.

Conservation status: Least concern (Foden et al., 2005).

Figure 2. A) *Rhodohypoxis baurii* var. *confecta* growing *in situ* near Sani Top. **B)** Longitudinal section of *Rhodohypoxis baurii* var. *confecta* flower showing the hidden reproductive structures.

Rhodohypoxis deflexa Hilliard & B.L.Burtt

Description: *Rhodohypoxis deflexa* is distinguished by its dwarf size, growing only 5 – 10 mm high and small, reddish-pink, or white flowers measuring 6 – 12 mm across, with the perianth disarticulating 2 – 3 mm above the ovary.

Distinguishing characters: The flower stalk bends over completely (after ferritization), thus placing the ovary flat on the soil next to the plant. The fruit is thin-walled and appears to rupture irregularly. Its leaves are heavily keeled, narrowly lanceolate, and smooth.

Habitat and distribution: Plants grow in marshy habitat, on the high Lesotho Plateau and near Barkly East in the Eastern Cape, between 2600 m.a.s.l. to 3200 m.a.s.l. Flowers from December to February.

Conservation status: Least concern (Foden et al., 2005).

Figure 3. A) *Rhodohypoxis deflexa* growing *in situ* near Sani Top. **B)** Close-up of *Rhodohypoxis deflexa* flowers.

Rhodohypoxis rubella (Baker) Nel

Description: *Rhodohypoxis rubella* grows up to 50 mm high, with thread-like, deeply keeled, smooth or slightly hairy leaves with small, bright pink, pale pink or white flowers that measure 8–15 mm across. The fruit is borne at or below ground level with a very definite epigynous beak to the ovary of up to nearly 1 cm long.

Distinguishing characters: The ovary is hidden amongst the leaf sheaths and the fruit is of the thin-walled, non-circumscissile type.

Habitat and distribution: Grow in moist gravelly soil around marshes in the Eastern Cape, KwaZulu-Natal, and Lesotho at 2500 m.a.s.l. to 3200 m.a.s.l. Flowers from November to February.

Conservation status: Least concern (von Staden et al., 2017).

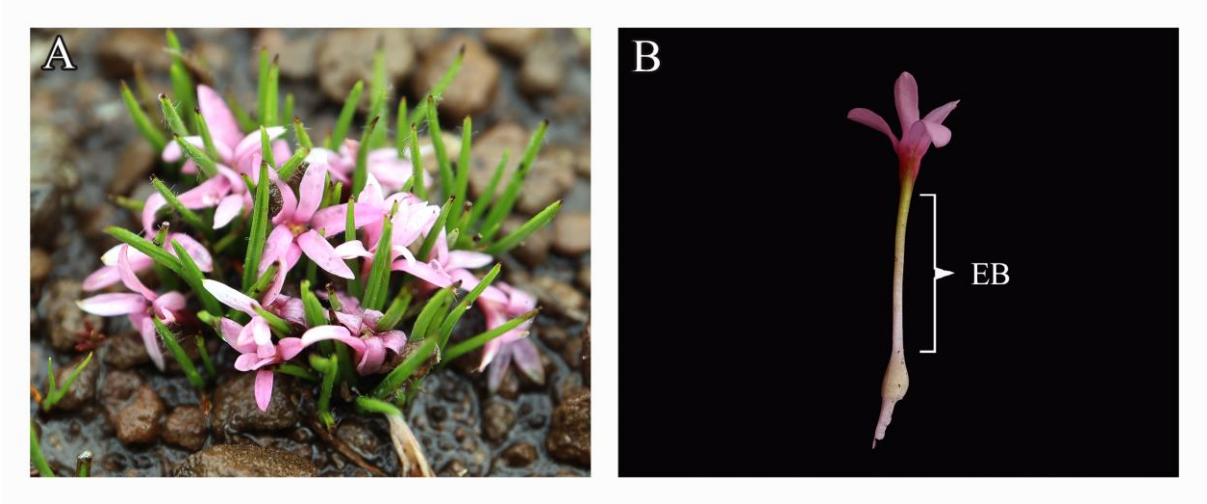

Figure 4. A) *Rhodohypoxis rubella* growing *in situ* near Sani Top. **B)** Isolated *Rhodohypoxis rubella* flower showing epigynous beak (EB) and the hidden subterranean ovary.

Rhodohypoxis incompta Hilliard & B.L.Burtt

Description: *Rhodohypoxis incompta* is recognized by its deeply keeled, thread-like leaves with very short hairs, and like *R. rubella*, the perianth segments that are resting close to the soil surface as well as a subterranean ovary that is covered by a very long epigynous beak of up to 37 mm long.

Distinguishing characters: Flower colour is always pale pink. The flowers measure 16 – 40 mm across.

Habitat and distribution: It occurs in wet grass tussocks on and around rock sheets at 2300 m.a.s.l. to 2900 m.a.s.l. Only known from around Sani Pass near the KwaZulu-Natal and Lesotho border. Flowers from October to January.

Conservation status: Rare, because of restrictive range (Singh et al., 2015).

Figure 5. A) *Rhodohypoxis incompta* growing *ex situ* near Sani Top. **B)** Isolated *Rhodohypoxis incompta* flower showing epigynous beak (EB) and the hidden subterranean ovary.

Rhodohypoxis thodiana (Nel) Hilliard & B.L.Burtt

Description: *Rhodohypoxis thodiana* is distinguished from other taxa by its flat, narrowly lanceolate and clear intramarginal vein, hairy leaves and scented pale pink solitary flowers. Individuals reach up to 30 mm high. The flowers measure 15 – 26 mm across and the ovary is covered by hairs.

Distinguishing characters: A well-developed pedicel always carries the 2 – 3 mm ovary above ground and the epigynous beak is 2 – 6.5 mm long.

Habitat and distribution: Restricted to Giant's Castle on the KwaZulu-Natal and Lesotho border, occurring on damp grassy slopes at 2600 m.a.s.l. to 3500 m.a.s.l. Flowers from November to January.

Conservation status: Rare, because of its restrictive range (Singh et al., 2015).

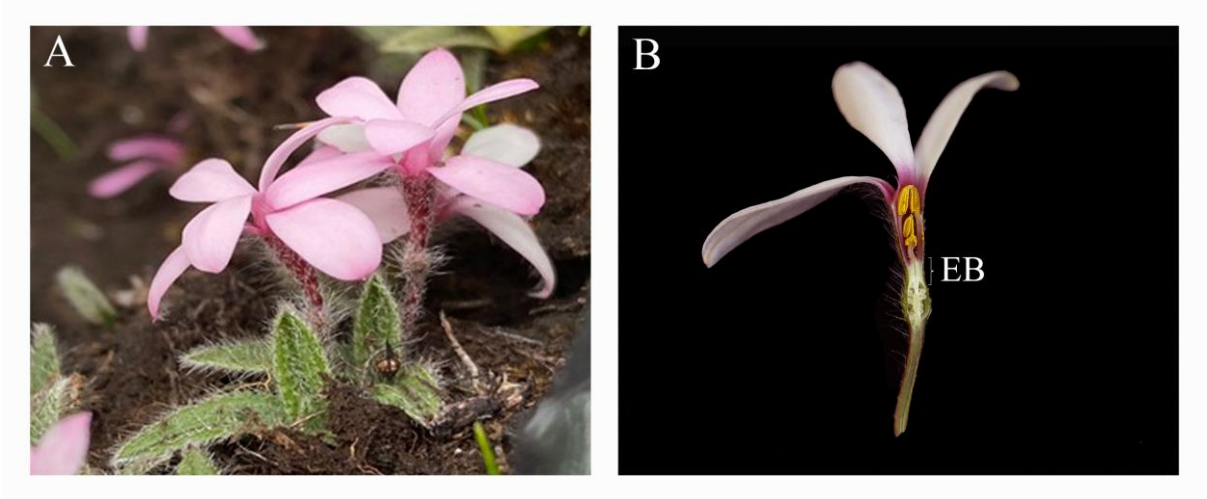

Figure 6. A) *Rhodohypoxis thodiana* growing *in situ* at Langalibalele Pass. Photo credit: T Hall. **B)** Longitudinal cross section of *Rhodohypoxis thodiana* showing the hidden reproductive structures and short epigynous beak (EB).

***Hypoxis* L.**

Hypoxis is a widespread genus of perennial geophytic herbs with hairy leaves and star-shaped yellow or rarely white flowers. The genus contains approximately 70 species that are found in the warmer regions of all continents except Europe and Antarctica (Singh, 2009). Based on the most recent systematic studies conducted by Singh (2009) and Niemann et al. (2023), there are currently 30 recognised species of *Hypoxis* found in South Africa – making it the most *Hypoxis* species-rich geographic region in the world.

In 1878, Baker described *Hypoxis membranacea* Baker and *Hypoxis parvula* Baker. *H. parvula* was described as having a single yellow flower lacking bracts. Baker subsequently changed this in 1896 and sunk *H. parvula* into *H. membranacea*. In the same year he described *H. brevifolia* Baker, which he documented as having a single yellow flower. Following this in 1914, Nel decided to sink *H. brevifolia* into *H. membranacea*. In 1976, Wood concluded that there are two distinct species, namely *H. membranacea* and *H. parvula*.

Then, in 1988, Hilliard & Burtt split *H. parvula* into two varieties – one with white and the other with yellow flowers (*Hypoxis parvula* Baker var. *albiflora* B.L.Burtt and *Hypoxis parvula* Baker var. *parvula*, respectively), and described the closely related species *Hypoxis limicola* B.L.Burtt. In 2009, Singh sunk *H. limicola* into *H. parvula* var. *parvula,* but this decision was overturned recently, with the re-instatement of *H. limicola* by Niemann et al. (2023) based on multiple new lines of evidence.

Putative hybridization between *Rhodohypoxis* and *Hypoxis parvula* has been documented various times in the wild – suggesting that there are few genetic barriers between the two genera (Hilliard & Burtt, 1978). Hybrid offspring between *Rhodohypoxis* and *Hypoxis* are generally referred to as ×*Rhodoxis* B. Mathew. In this study, the focus was on *H. parvula* var. *parvula* because this variety is the only one that grows high enough to be found in sympatry with some of the alpine *Rhodohypoxis* taxa discussed above.

Hypoxis parvula var. *parvula* Baker

Description: *Hypoxis parvula* var. *parvula* is a small, delicate, sparsely hairy herb, up to 80 mm high, growing singly on the slopes of the Drakensberg.

Distinguishing characters: *Hypoxis parvula* var. *parvula* is distinguished by having soft leaves, yellow flowers with linear yellow filaments, a long style, and a capitate stigma. It is morphologically most similar to *H. limicola* from which it can be distinguished by having tepals that are (7–)8–10 mm long (not 2–6 mm long), an inconspicuous (not conspicuously swollen) articulation between scape and pedicel, and a single 1–4 mm long bract (not one or two 5–7 mm long bract(s)) when present. It also resembles *H. uniflorata* Markötter in its habit, thin leaves, long slender inflorescences, and delicate flowers. It can, however, be easily distinguished from *H. uniflorata* in having lanceolate (not linear) leaves, filiform (not subulate) anther filaments, and a spherical (not pyramidal) stigma (Niemann et al., 2023).

Habitat and distribution: Growing singly on the slopes of the Drakensberg in KwaZulu-Natal and nearby mountains, usually above 1980 m.a.s.l. and reaching 2500 m.a.s.l.

Conservation status: Least concern (Foden et al., 2005)

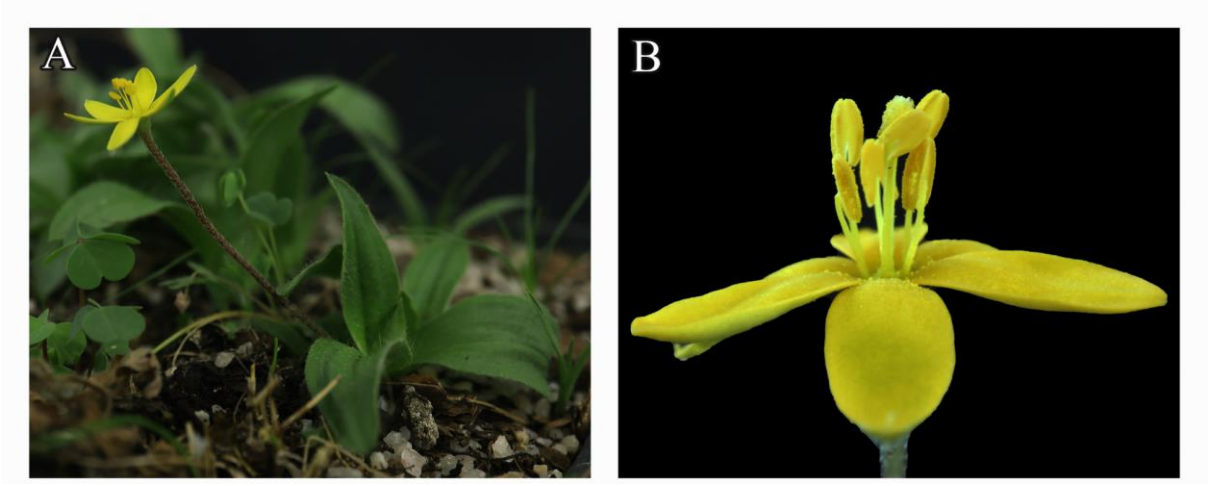

Figure 7. A) *Hypoxis parvula* var. *parvula* growing *ex situ* from Sentinel Peak. **B)** Close-up of a *Hypoxis parvula* var. *parvula* flower showing the reproductive structures.

STUDY SITES

The 40,000 km² high-altitude range of mountain peaks and escarpment plateau surrounding the eastern heartland of southern Africa is known as the Drakensberg. (Carbutt & Edwards, 2003). The Drakensberg is the highest mountain system in Africa, south of Mount Kilimanjaro, and the only southern African mountain system with an alpine zone (Carbutt 2019; Nel & Sumner 2008). The region has a temperate climate, with annual rainfall ranging between c. 640 mm to 1800 mm in the summer. The shallow soils of the summit plateau are frequently wet during the summer and prone to freeze-thaw cycles throughout the winter (Killick, 1994). On a broad scale, altitudinal, climatic, topographic, and edaphic gradients can account for both floristic and vegetative diversity. At a finer scale micro-habitat, when taken as a whole, provide suitable ecological niches for the coexistence of numerous species (Carbutt & Edwards, 2003).

The three study sites were identified based on previously observed *Rhodohypoxis* hybrid zones within the borders of South Africa. All sites have *R. baurii* var. *confecta* as one of the co-occurring species. Near Sani Top was selected because it boasts the most co-occurring *Rhodohypoxis* species of all the study sites: *R. deflexa, R. incompta, R. baurii var. confecta, R. rubella*, but excludes any co-occurring *Hypoxis* species. Langlibalele Pass were selected because it has the rare *R. thodiana* co-occuring with *R. baurii* var. *confecta* and *H. parvula* var. *parvula.* The third location of specific interest was Sentinel Peak because a possible ×*Rhodoxis* hybrid (Singh 555 in NH) was found there (Singh, 2009). This is also the locality where *R. baurii* var. *confecta* (Singh 554 in NH) and *H. parvula* var. *parvula* (Singh 465 in NH) were collected for the molecular phylogenetic study of Kocyan et al. (2011) to elucidate generic limits within the Hypoxidaceae. It should be noted that Singh 554 was mistakenly identified as *R. baurii* var. *baurii*. This variety is not known to grow in the northern Drakensberg or at Sentinel Peak (Hilliard & Burtt, 1978). Furthermore, the photograph of the specimen in Singh (2009) shows both white and pink flowers on the same plant, which is diagnostic for *R. baurii* var. *confecta*.

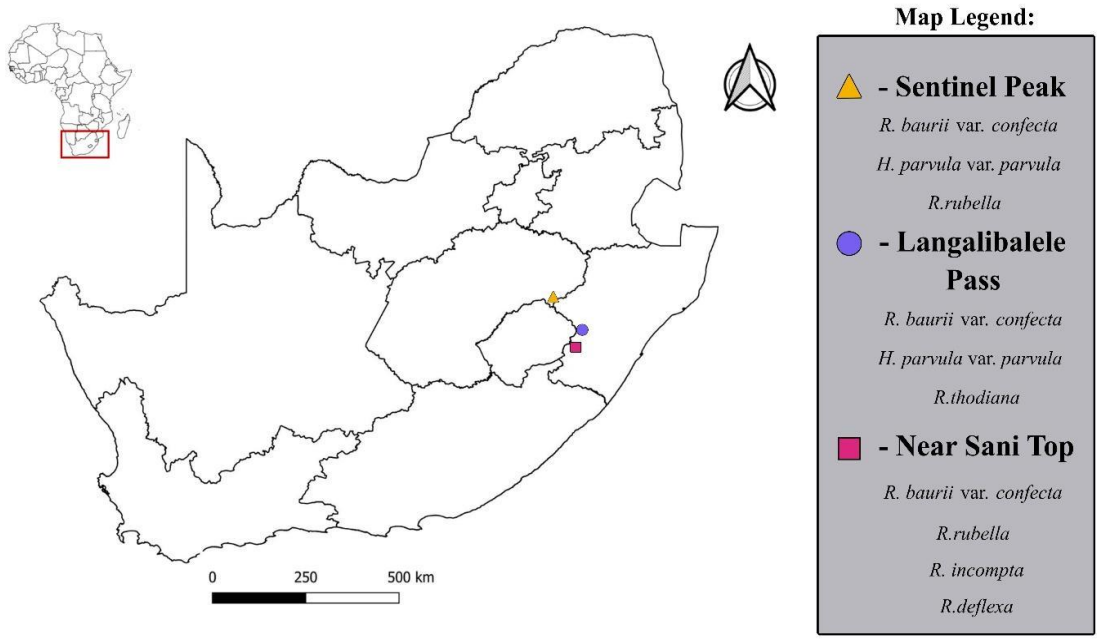

Figure 8. Map of South Africa showing sampling locations used in this study.

AIMS AND OBJECTIVES

The primary aim of this study was to determine patterns of hybridization between the high altitude *Rhodohypoxis* species as well as between *R. baurii* var. *confecta* and *H. parvula* var. *parvula*. A second related aim was to contribute new data that might help delineate/solidify genus and species boundaries within this group.

To address these aims, I addressed the following objectives:

i) Test if hybridization occurs between *R. deflexa, R. rubella, R. incompta, R. thodiana, R. baurii* var. *confecta* and *H. parvula* var. *parvula* at three different natural study sites using microsatellites to estimate the extent of gene flow.

ii) Evaluate whether the genetic clusters are consistent with morphology-based designations.

iii) Conduct controlled greenhouse crosses to test if there are any post-pollination reproductive barriers between *R. baurii* var. *confecta* and *H. parvula* var. *parvula* from Sentinel Peak.

CHAPTER 2: MATERIALS & METHODS

MORPHOLOGICAL METHODS

Morphometric data collection

Plants were measured in their natural habitat immediately before material was collected for the genetic section of this study (see below for details). All taxonomic assignments were based on the monograph of Hilliard & Burtt (1978). Nine continuous morphological characters (floral and vegetative) were assessed (see Figure 9 and Table 1) across the following 136 mature, flowering *Rhodohypoxis* individuals: *R. baurii* var. *confecta* (N = 40), *R. deflexa* (N = 25), *R. rubella* (N = 16), *R. incompta* (N = 29), *R. thodiana* (N = 13), and putative hybrids (N = 13). All the relevant data were collected using a Grip 150 mm Digital Vernier Calliper with an accuracy of 0.01 mm. To reduce errors, all measurements were taken by the same person and with the same instrument.

Table 1. List of morphological characters studied in *Rhodohypoxis*. All variables were measured in millimetres (mm).

Character acronym:	Description of the character:
Outer tepal length (OTL)	Measured from the top of the perigone tube to tip of outer tepal.
Outer tepal width (OTW)	Measured at widest point of outer tepal. Basal claw wasn't included in the measurement.
Inner tepal length (ITL)	Measured from the top of the perigone tube to tip of inner tepal. Basal claw wasn't included in the measurement.
Inner petal width (ITW)	Measured at widest point of inner tepal.
Scape plus pedicel length (SL+PL)	Measured from the point of exsertion from the leaf sheaths at ground level to base of receptacle.
Epigynous beak length (EBL)	Measured from the base of the style to the top of the ovary.
Plant height (PH)	The above-ground height of mature flowering individuals.
Leaf lamina width (LW)	Measured at widest point of widest leaf.
Leaf lamina length (LL)	Longest leaf measured from point of attachment to leaf tip of longest leaf.

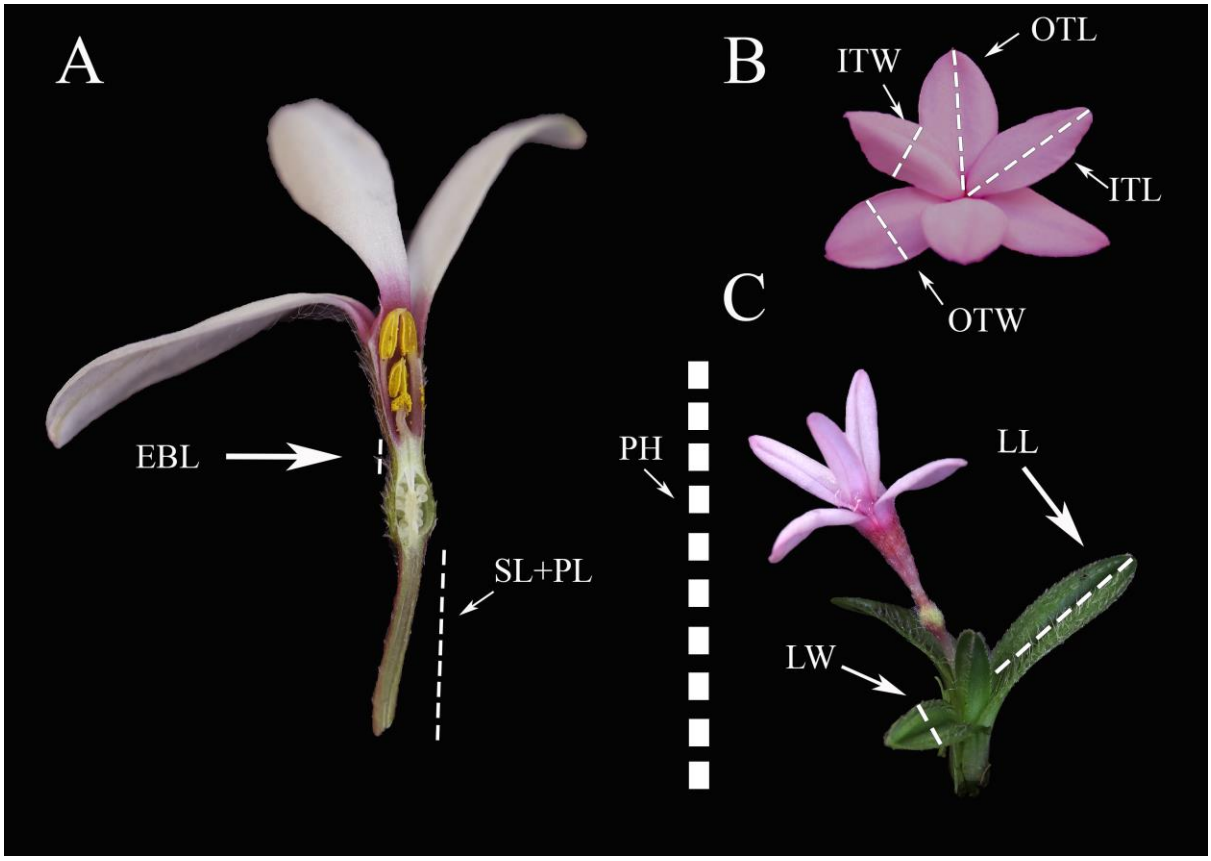

Figure 9. Measurements taken for the morphometric analysis. **A)** A longitudinal section of a *R. thodiana* flower to illustrate how the epigynous beak length (EBL) and the scape plus pedicel length (SL+PL) was measured. **B)** A *R. baurii* var. *confecta* flower to illustrate how the outer tepal length (OTL), outer tepal width (OTW), inner tepal length (ITL), and inner tepal width (ITW) was measured. **C)** A mature flowering *R. thodiana* plant to illustrate how plant height (PH), leaf lamina length (LL) and leaf lamina width (LW) was measured.

Morphometric data analysis

All morphometric analyses were conducted in R v4.3.3 (RStudio Team, 2022). A Principal Components Analysis (PCA) was done, because all traits were quantitative. Prior to analysis though, the character matrix was log transformed to stabilize variances and normalize the distribution using the '*log*' function in the base R '*stats*' package (version 4.3.0). The PCA was then performed on the transformed dataset using the '*prcomp'* function in the same package. To visualize the PCA results, a biplot was generated using the '*ggbiplot*' function from the '*ggbiplot*' package (version 0.55). Finally, the functions '*get_pca_var'* and '*fviz_contrib*' from the package '*factoextra*' (version 1.0.7) was used to visualize the contribution of each character to principal component (PC) 1 and 2.

Seed morphology

To observe and document seed surface morphology, five dried seeds were collected for each of the five *Rhodohypoxis* taxa and from putative hybrid individuals in the University of the Witwatersrand living collection. The seeds were imaged by scanning electron microscopy (SEM) using a Phenom ProX Desktop SEM system (Thermo Fisher Scientific, Einhoven, Netherlands). Unfixed/uncoated samples were placed on a carbon disk stuck to an aluminium stub and placed on the 'Charge Reduction Sample Holder'. SEM images were acquired using a high sensitivity multi-mode backscatter electron (BSE) detector at 5 kV and at 230×/2200× magnification. The emphasis on seed surface micromorphology in this study stems from its recognised taxonomic significance in *Hypoxis* (Brackett, 1923; Henderson, 1987; Herndon 2002; Wiland-Szymańska, 2006, Niemann et al., 2023) – which is closely related to *Rhodohypoxis* (Kocyan et al., 2011). The "secondary sculpture", which refers to periclinal cell wall ornamentation, and the "primary sculpture" of the seed surface, which refers to epidermal cell patterns and shapes (Barthlott, 1981), were of particular significance in *Hypoxis* – and were therefore also assessed here.

ASSESSMENT OF GENE FLOW AMONG TAXA

Plant material collection

In 2022, leaf material was collected from mature flowering individuals of all the focal *Rhodohypoxis* species and putative hybrids present at Sentinel Peak (latitude: -29.72665, longitude: 28.89085; *R. baurii var. confecta;* n = 15; *R. rubella*, n = 15; putative hybrids, n = 3), near Sani Top (latitude: -29.585141, longitude: 29.286038; *R. deflexa*, n = 25*; R. rubella*, n = 25*; R. incompta*, n = 25 *; R. baurii* var. *confecta*, n = 25; putative hybrids, n = 6) and Langalibalele Pass (latitude: -29.279756, longitude: 29.436558; *R. thodiana*, n = 25; *R. baurii* var. *confecta*, n = 25; putative hybrids, n = 14). When present, leaf material from *H. parvula* var. *parvula* individuals were also collected (Sentinel Peak, n = 20; Langalibalele pass, n = 7). Upon collection, all leaf samples were immediately preserved and stored in silica gel until DNA extraction could be performed.

DNA extraction

DNA was extracted from the collected silica-dried leaf material (10–20 mg). A modified version of the 2% CTAB extraction protocol (Doyle & Doyle, 1987; Cullings, 1992) available from the Soltis lab (https://www.floridamuseum.ufl.edu/soltis-lab/protocols/) was

used. Briefly, the ß-mercaptoethanol concentration of the extraction buffer was increased from 0.5% to 1.5%, the incubation time of the samples during the lysis step was increased from 60 minutes to 180 minutes, and the step requiring the addition of cold 7.5 M ammonium acetate prior to the alcohol precipitation step was removed. A Thermo Fischer Scientific Nanodrop one UV-vis spectrophotometer (ThermoFisher Scientific, MA, USA) was used to determine the quantity and quality of the extracted DNA, and the samples were accordingly normalised to a final concentration of 50 ng/uL.

PCR and fragment analysis

The individuals were genotyped utilizing the six most variable microsatellite markers previously developed for *R. baurii* by AllGenetics (A Coruña, Spain) using the protocols described in Fererria et al. (in prep). The oligonucleotide tails used were the universal sequences CAG (CAG TCG GGC GTC ATC A), and T3 (AAT TAA CCC TCA CTA AAG GG). The two oligonucleotides were labelled with the FAM dye, and the NED dye, respectively. Fluorescent microsatellite PCR amplifications were conducted as single-plex reactions.

The amplification process was performed using a BioRad T100™ thermal cycler (Bio-Rad laboratories, CA, USA) following a precise cycling profile: initial denaturation at 95°C for 1 minute, succeeded by 33-35 cycles of denaturation at 95°C for 20 seconds, annealing at 57°C for 60 seconds, and extension at 68°C for 45 seconds. A final extension step at 68°C for 5 minutes concluded the process.

The efficacy of the amplification was evaluated through gel electrophoresis. Here, 3µl of 6x purple loading dye (New England BioLabs, MA, USA) was mixed with 5 µl of the PCR product and loaded onto a 4% agarose gel to which ethidium bromide was added (0.5ug/mL). A 50bp DNA ladder (New England BioLabs, MA, USA) was also loaded to assess the fragment size of the PCR products. The gel was then run at 65 volts for 20 minutes. Finally, 5 µl of successfully amplified PCR product per sample was sent for fragment analysis at the Central Analytical Facility (CAF), Stellenbosch.

Table 2. Characteristics of the six most polymorphic microsatellite markers developed for the *R. baurii* varieties by AllGenetics (AllGenetics & Biology SL, A Coruña, Spain).

Locus	Motif	size (bp)	Forward primer sequence	Reverse primer sequence	Label	Tails
AG_Rba_182	AG	144	AATGGCTGACACCAACTTCC	GGAATGACATCAGCCCAAAC	FAM	CAG
AG_Rba_290	AG	121	ACAGGAGTGCATCCTTCGTT	GGGTTATGCACATGGGTAGC	FAM	CAG
AG_Rba_473	AG	153	ATCTCGATGGTGATCCAACC	TAATCCAAAGCGGAGACCAT	FAM	CAG
AG_Rba_165	AAC	132	CGACGAAATGGTCGACACTA	AGCCCTTCTTCTCCGACTTC	NED	T3
AG_Rba_307	AG	129	ACCCATTGTTCCTCCTTCCT	AATGAGTTGCAGTGCACCAA	NED	T3
AG_Rba_448	ATC	91	CCATAGCTGATCCCATTGCT	GGTGTTCCATTGGACGTCAT	NED	T3

Microsatellite data analyses

The resulting chromatograms were scored manually using the microsatellite plugin for Geneious Prime Version 11.0.6+10 (Kearse et al., 2012) and four data matrices were compiled: one with all the scored data for the three study sites combined, and one for each of the three separate study sites. Prior to analysis, the polyploid Sentinel Peak *H. parvula* var. *parvula* microsatellite data was diploidized using Genodive version 3.05 (Meirmans, 2020). This software was also used to determine the observed number of alleles (A_o), effective number of alleles (A_e), observed individual heterozygosity (H_o), heterozygosity in population (H_s), and inbreeding coefficient (G_{is}) for the complete combined data matrix. The fixation index (F_{ST}) was also calculated for this dataset using Genodive, but a heat map of this data was created in R v4.3.3 (RStudio Team, 2015) using the '*ggplot2*' package.

The analysis of admixture among the species at each study site was performed in STRUCTURE version 2.3.4 (Pritchard et al., 2000). Each of the three separate data matrices (corresponding to a particular study sites) were run with a burn-in period of 25 000 and a further 150 000 repeats using the admixture model and correlated allele frequencies. This was repeated 5 times for different values of K (K = 1 – 5). Admixture plots were then created

based on the STRUCTURE results and the optimal cluster value was determined using the CLUMPAK software (Kopelman et al., 2015).

Discriminant Analysis of Principal Components (DAPC) was conducted for each of the four data matrices in R v4.3.3. The genetic data was imported into RStudio as a genclone object *and the 'dapc*' function from the '*adegenet*' package (version 2.1.10; Jombart et al., 2010) together with the '*ggplot2*' package were used to create DAPC plots. This multivariate method combines elements of Principal Component Analysis (PCA) and Discriminant Analysis (DA) and is particularly useful for analysing genetic data where groups are known.

GREENHOUSE CROSSING EXPERIMENT

Flowering phenology

Due to the recent re-circumscription of *H. parvula* var. *parvula* (Niemann et al., 2023), it seemed pertinent to assess the extent of overlap in flowering phenology between this taxon and *R. baurii* var. *confecta*. Accordingly, a comprehensive, carefully curated dataset of herbarium specimens and research grade iNaturalist observations were compiled for *H. parvula* var. *parvula* and *R. baurii* var. *confecta* using the Global Biodiversity Information Facility (GBIF; https://www.gbif.org/) and the Botanical Database of Southern Africa (BODATSA; https://posa.sanbi.org/) (See Table A1 in the Appendix for full list of specimens examined). Following taxonomic verification using the relevant literature (Hilliard & Burtt, 1978; 1988; Niemann et al., 2023), a total of 90 records (encompassing collections across southern Africa between 1988 and 2022) could be used to construct a flowering phenology chart.

Plant collection

Approximately twenty-five individuals of *H. parvula* var. *parvula* and ninety individuals of *R. baurii* var. *confecta* were collected from Sentinel Peak carpark between 2016 and 2021. The individuals were potted up and maintained in the closed rooftop glass greenhouse at the University of the Witwatersrand, Johannesburg, South Africa. Before being used in the crossing study, the plants were given a minimum of one flowering season to acclimatise to controlled watering and temperature conditions, which entailed maintaining temperatures within the range of 10–30 °C in a 24-hour cycle, under natural light conditions. The plants were irrigated using an automated sprinkler system, receiving a total of ~200 ml of water per

day spread out over three separate waterings (the first at 07h00, the second at 12h30, and the third at16h30).

Pollination and seed set

Plants included in the crossing study (*R. baurii var. confecta;* n = 15; *H. parvula* var. *parvula*; n = 15) were selected from the available plants that flowered during 2022 flowering season of the greenhouse plants (2022/10/29 - 2023/01/21). The study involved a total of 203 crosses and self-pollinations, with an average of 33 crosses per cross-type, ranging from 25 to 48 crosses. Pollinations and flower emasculations were performed using forceps. To prevent unintended pollinations, these forceps were treated with 95% isopropyl alcohol and wiped down with a Kim wipe between each cross/emasculation.

Self-pollinations of *R. baurii* var. *confecta* and *H. parvula* var. *parvula* individuals were carried out by removing all dehiscent anthers of a single flower, then transferring the pollen from the removed anthers onto the stigma of the same flower – taking special care not to damage the stigma. Intraspecific and intergeneric crosses were performed between *R. baurii* var. *confecta* and *H. parvula* var. *parvula* individuals by first emasculating the immature flower buds of the seed parent prior to performing the relevant cross to prevent self-pollination. The intended pollination was then performed by transferring the pollen of the appropriate pollen parent onto the stigma – once again taking special care not to damage the stigma.

To serve as negative controls, but also to examine the possible presence of apomixis, the buds of five flowers from five individuals of each taxon were emasculated without pollination. After the respective crosses/emasculations were complete, flowers were carefully covered using empty tea bags and left to fruit. Once the fruits reached maturity (the thin-walled capsules underwent circumcessile dehiscence), they were removed, and the included viable seeds were counted.

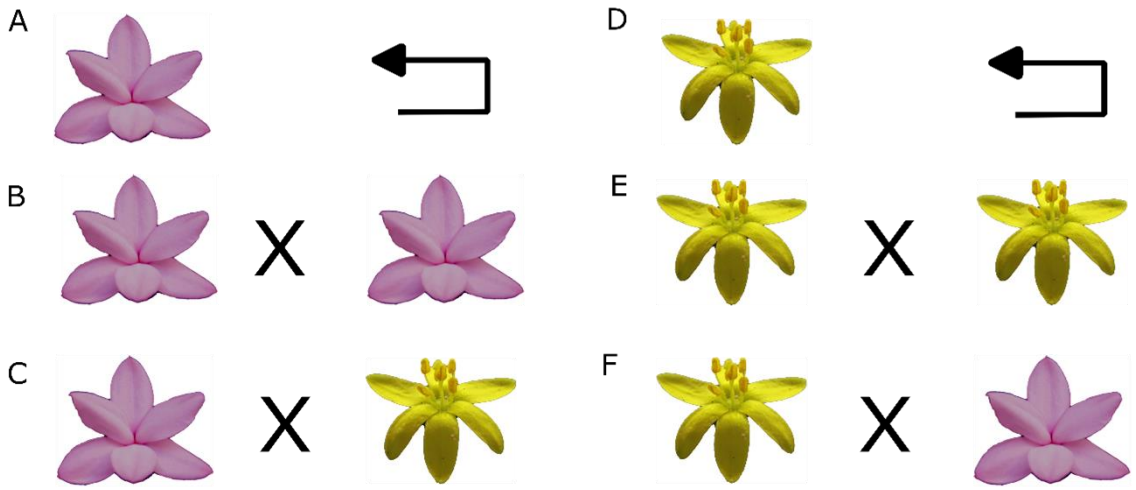

Figure 10. Graphic representation of controlled greenhouse hand self-pollinations and crosses. **A)** R. *baurii* var. *confecta* self-pollinations. **B)** R. *baurii* var. *confecta* intraspecific crosses. **C)** *R. baurii* var. *confecta* intergeneric cross with *H. parvula* var. *parvula* as pollen doner. **D)** *H. parvula* var. *parvula* self-pollinations. **E)** *H. parvula* var. *parvula* intraspecific crosses. **F)** *H. parvula* var. *parvula* intergeneric cross with as *R. baurii* var. *confecta* pollen donor.

Germination procedure

Prior to germination experiments, seeds were stored dry in small brown envelopes at room temperature for no longer than three months post-harvest. Seeds were then germinated in a controlled growth chamber (Labcon / LTGC40/ 27 Pillans St, Chamdor, Johannesburg) at the University of the Witwatersrand, South Africa set to a constant 27°C with a 12:12 hr light: dark photoperiod to simulate summer conditions. The seeds were sown in sterile 90 mm Petri dishes containing filter paper saturated with reverse osmosis water. A maximum of 34 seeds were sown per Petri dish to prevent overcrowding. The planted Petri dishes were monitored every three days for a total of 31 days from the time of sowing. The germination criterion was defined as the visible protrusion of the radicle from the seed. If there was no evidence of a radicle after 31 days, it was deemed ungerminated.

Seed set and germination data analysis

All seed set and germination data analyses were conducted in R v4.3.3 (RStudio Team, 2022) using the base R '*stats*' package (version 4.3.0). First, a Kruskal-Wallis rank sum test was performed to assess the potential differences in seed set across various hand pollination-types by using the '*kruskal.test*' function. Then, a Generalised Linear Model (GLM) analysis was

conducted with the function '*glm*' on the relationship between pollination type and germination success.

DNA index estimation

Flow cytometry (FC) was used to assess the DNA index of all the plants from Sentinel Peak car park that were involved in the greenhouse crossing experiment as well as F1 hybrid plantlets (*H. parvula* var. *parvula* × *R. baurii var. confecta*; n = 4). The FC analysis was conducted according to the protocol described by Doležel et al. (2007) on a Sysmex Cyflow Space flow cytometer (Sysmex South Africa Ltd, Randburg, South Africa) equipped with a UV LED chip for 4',6-diamino-2-phenylindole (DAPI) excitation available at the H.G.W.J. Schweickerdt Herbarium (PRU), University of Pretoria.

Briefly, for each FC run, approximately 0.5 cm^2 of internal standard leaf tissue (*Oxalis articulata* Savigny; 2C = ~0.91pg) and an equal amount of the query sample leaf tissue were finely chopped together using a new, unused razor blade in 1ml of Otto I buffer (0.1 M citric acid, 0.5% Tween 20). The resulting slurry was thoroughly mixed using a wide bore pipette tip and passed through a 42 µm disposable mesh nylon filter. The solution was then left to incubate at room temperature (RT) for a minimum of 20 minutes. After the initial incubation step, 1 ml of Otto II buffer (0.4 M $Na_2HPO_4 \cdot 12\ H_2O$) containing DAPI dye (at final concentration 4 µg/ml) and ß-mercaptoethanol (2 µg/ml) was added to the solution. The samples were then left to incubate for a minimum of 10 minutes at RT. Then, fluorescence intensity was measured on the flow cytometer. The flow rate was adjusted until there was on average 25 events per second and then the fluorescence of ~5,000 particles were recorded.

Histograms were evaluated using FloMax software (Partec GmbH, Münster, Germany). Peaks were gated manually, and for both the standard and the sample, the coefficient of variation (CV; Doležel & Bartoš 2005) as well as the mean fluorescence intensities (MFI) of the G0/G1 peak were recorded. The flow cytometer was adjusted so that the G0/G1 peak of the internal standard was located at channel 100. The DNA index (ratio) as per Sliwinska et al. (2022) was then calculated for each sample (Sample G0/G1 peak MFI ÷ Standard G0/G1 peak MFI).

Pollen viability assay and measuring

Anthers were removed from the *H. parvula* var. *parvula* and *R. baurii* var. *confecta* individuals from the Sentinel Peak that were involved in the greenhouse crossing experiment.

Additionally, anthers were also collected from three known tetraploid *R. baurii* var. *platypetala* individuals from Minerva Private Nature Reserve (Mtileni et al., 2024*;* DNA index=~4.03) as well as from three *H. parvula* var. *parvula* individuals from Monk's cowl with the lowest known DNA index for the species (Niemann et al., 2023; DNA index=~1.95). The anthers were harvested before anthesis at day 3 after the flowers had opened (when pollen was mature but anthers not yet dehiscent) and fixed in microcentrifuge tubes containg ~200µL Carnoy's fixative (60% chloroform, 30% ethanol, 10% acetic acid). The microcentrifuge tubes were refrigerated and stored for a minimum of four months and a maximum of six months prior to microscope slide preparation.

Then, a modified Alexander's stain (Alexander, 1969) was prepared which dyes viable pollen grains magenta-red and inviable pollen grains blue/green. The stain contained 10 ml of 95% ethanol, 1 ml of malachite green (1% in 95% Ethanol), 5 ml of fuchsin acid (1% in water), 0.5ml orange G (1% in water), 5 g phenol, 2 ml glacial acetic acid, 25 ml glycerol, and 50 ml distilled water (Peterson et al., 2010).

The microcentrifuge tubes (containing the fixed anthers) were vortexed for 2 minutes to release the pollen from the anthers. Then, 100 µl of the pollen grain suspension was added to a slide and left for 10 minutes for the fixative to evaporate. Thereafter, 23 µl of the prepared Alexander stain was added to the slide, mounted with a glass coverslip, and sealed with clear nail polish. The prepared slides were observed under a Zeiss Axio Imager M2 Compound Microscope (Zeiss, Cambridge, UK) and photographed with a Zeiss Axiocam 506 colour digital camera at a magnification of ×100.

A minimum of five chosen fields of view were selected from each slide, photographed from left to right in a grid like pattern, and the number of viable and inviable pollen grains were counted (a minimum of 129 and maximum of 1124 pollen grains per plant were assessed). Percentage of pollen fertility (PP) was calculated as follows: PP = (Total number of fertile pollen count/Total number of pollen grains counted) x 100. The same pollen viability photographs were used to measure the diameter (at the wides part of the grain) of at least 129 viable pollen grains per sample using the Fiji/ImageJ software (Schindelin al., 2012).

Pollen viability and size data analysis

The pollen viability and size data analyses were conducted in R v4.3.3 (RStudio Team, 2015) once again using the base R '*stats*' package (version 4.3.0). The pollen viability between *H. parvula* var. *parvula* and *R. baurii* var. *confecta* was compared with a Welch Two Sample t-test by using the '*t.test'* function with the argument "var.equal" set to "FALSE". A Spearman's rank correlation test was also conducted using the '*cor.test*' function with the argument method set to "spearman" to investigate the relationship between DNA index levels and means pollen size in the *R. baurii* var. *confecta* and *H. parvula* var. *parvula.*

CHAPTER 3: RESULTS

MORPHOLOGICAL DATA

Morphometrics

The 2-dimensional (2D) scattergram resulting from PCA of the morphometric dataset that each of the five high altitude *Rhodohypoxis* species created distinct clusters outlined by the respective 80% confidence ellipses (see Table 3 for summary statistics for each trait measurement by species). There is only partial overlap between the *R. baurii* var. *confecta* and the *R. thodiana* ellipses. However, the inclusion of an 80% confidence ellipse around the putative hybrids (not shown) partially overlaps with *R. baurii var. confecta, R. incompta* and *R. deflexa*, but completely overlaps with *R. thodiana*. PC1 explains 48.7% of variation and PC2 explains 19.2 %.

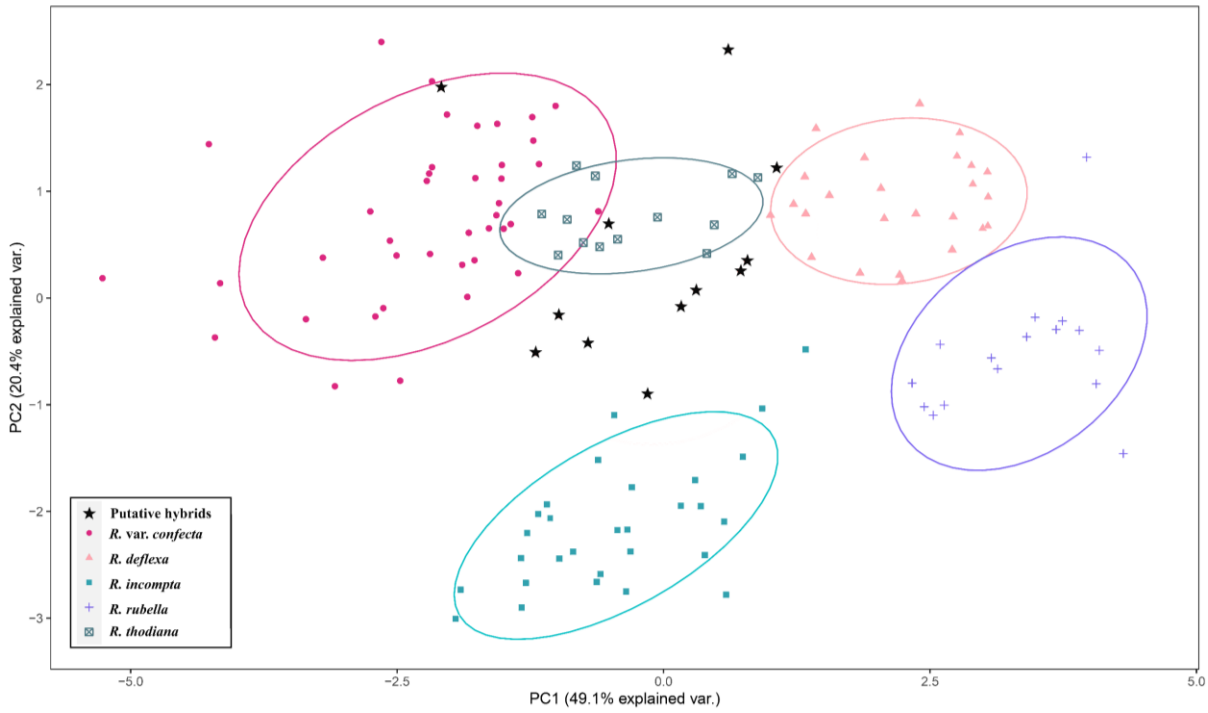

Figure 11. Principal Component Analysis (PCA) based on nine morphological characters measured for 123 individuals belonging to one of the five high-altitude *Rhodohypoxis* species as well as 13 putative hybrid individuals. The five species are delineated by 80% confidence ellipses. PC1 explains 48.7 % of the variation, while PC2 explains 19.6 % of the variation.

Table 3. Summary statistics for each character by species. Number of observations (n), minimum (min) and maximum (max) values, mean values, standard deviation (st. dev.), and 5th and 95th percentiles are reported. OTL = outer tepal length; OTW = outer tepal width; IPL = inner tepal length; IPW = inner tepal width; LL = leaf lamina length; LW = leaf lamina width; PH = plant height; SL+PL = scape plus pedicel length; EBL = epigynous beak length.

Taxon	Parameter	OTL (mm)	OTW (mm)	IPL (mm)	IPW (mm)	LL (mm)	LW (mm)	PH (mm)	SL+PL (mm)	EBL (mm)
R. baurii v. *confecta* n=40	min–max	7.43 – 15.41	4.15 – 9.65	6.77– 13.79	3.57 – 9.62	12.06 – 46.57	2.76 – 7.74	6.58 – 57.40	11.90 – 55.63	0.80 –1.10
	mean/st. dev.	10.03 / 1.71	6.16 / 1.32	9.09 / 1.69	5.35 / 1.42	24.16 / 8.81	4.31 / 1.12	21.66 / 10.72	22.40 / 10.44	0.95 / 0.067
	5-95 quantiles	8.10 – 12.70	4.78 – 8.57	7.18– 11.52	3.91 – 8.24	14.33 – 42.80	2.80 – 6.20	8.15 – 38.18	12.45 – 46.34	0.90 – 1.10
R. deflexa n=25	min–max	2.74 – 5.11	1.65 – 3.29	2.01– 3.64	1.02 – 2.82	15.23 – 35.50	1.81 – 7.31	15.01 – 41.16	3.45 – 12.62	0.80 – 1.10
	mean/st. dev.	3.67 / 0.57	2.16/ 0.40	2.76 / 0.44	1.90 / 0.37	21.67 / 5.10	3.70 / 1.27	23.91 / 6.59	7.59 / 2.58	0.94 / 0.08
	5-95 quantiles	2.98 – 4.66	1.67 – 2.79	2.07– 3.37	1.51– 2.52	15.50 – 30.79	2.09 – 5.26	15.74 – 34.94	4.52 – 11.91	0.80 – 1.10
R. incompta n=29	min–max	5.82 – 16.24	2.49– 5.65	5.47 – 13.35	1.79 – 4.37	11.43 – 49.82	1.08 – 2.77	14.01 – 41.66	8.20 – 20.56	13.65 – 19.78
	mean/st. dev.	10.30 / 2.65	4.06 / 0.90	8.84 / 1.96	2.91 / 0.63	27.16 / 9.67	1.73 / 0.41	27.69 / 8.29	14.32 / 3.83	16.52 / 1.74
	5-95 quantiles	6.44 – 13.72	2.92– 5.40	5.66 – 11.34	2.00 – 3.77	12.63 – 45.11	1.17 – 2.38	14.17 – 39.53	8.93 – 20.04	14.23 – 19.58
R. rubella n=16	min–max	2.08 – 5.21	1.20 – 2.54	1.79– 4.87	0.52– 2.00	8.35 – 21.67	0.47 – 1.91	6.23 – 24.46	6.02 – 8.68	7.35 – 11.79
	mean/st. dev.	3.57 / 0.98	1.80 / 0.36	3.15 / 0.80	1.51 / 0.38	15.18 / 3.65	1.23 / 0.33	16.72 / 4.42	7.33 / 0.80	9.16 / 1.37
	5-95 quantiles	2.12– 5.02	1.26 – 2.27	2.03– 4.33	0.90 – 1.99	10.31 – 21.01	0.79 – 1.74	11.29 – 23.93	6.11– 8.41	7.47 – 11.45
R. thodiana n=13	min–max	6.96 – 10.32	3.00 – 5.19	6.58 – 10.22	2.27 – 4.44	13.53 – 22.51	4.43 – 6.19	13.27 – 25.72	3.01 – 10.46	1.88 – 6.15
	mean/st. dev.	9.24 / 0.90	3.82 / 0.69	8.48 / 1.07	3.24 / 0.79	17.55 / 2.96	5.16 / 0.58	19.54 / 3.81	7.15 / 1.91	3.55 / 1.26
	5-95 quantiles	7.83 – 10.21	3.11 – 4.97	6.79 – 9.83	2.31 – 4.35	14.17 – 21.77	4.55 – 6.14	15.16 – 25.48	3.82 – 9.63	1.97 – 5.62

Tepal characters and scape plus pedicel length contributed the most to PC 1, while the epigynous beak length character and the leaf lamina width contributed most to PC 2 (Figure 12). Only the leaf lamina length had no substantial contribution to either of the first two principal components.

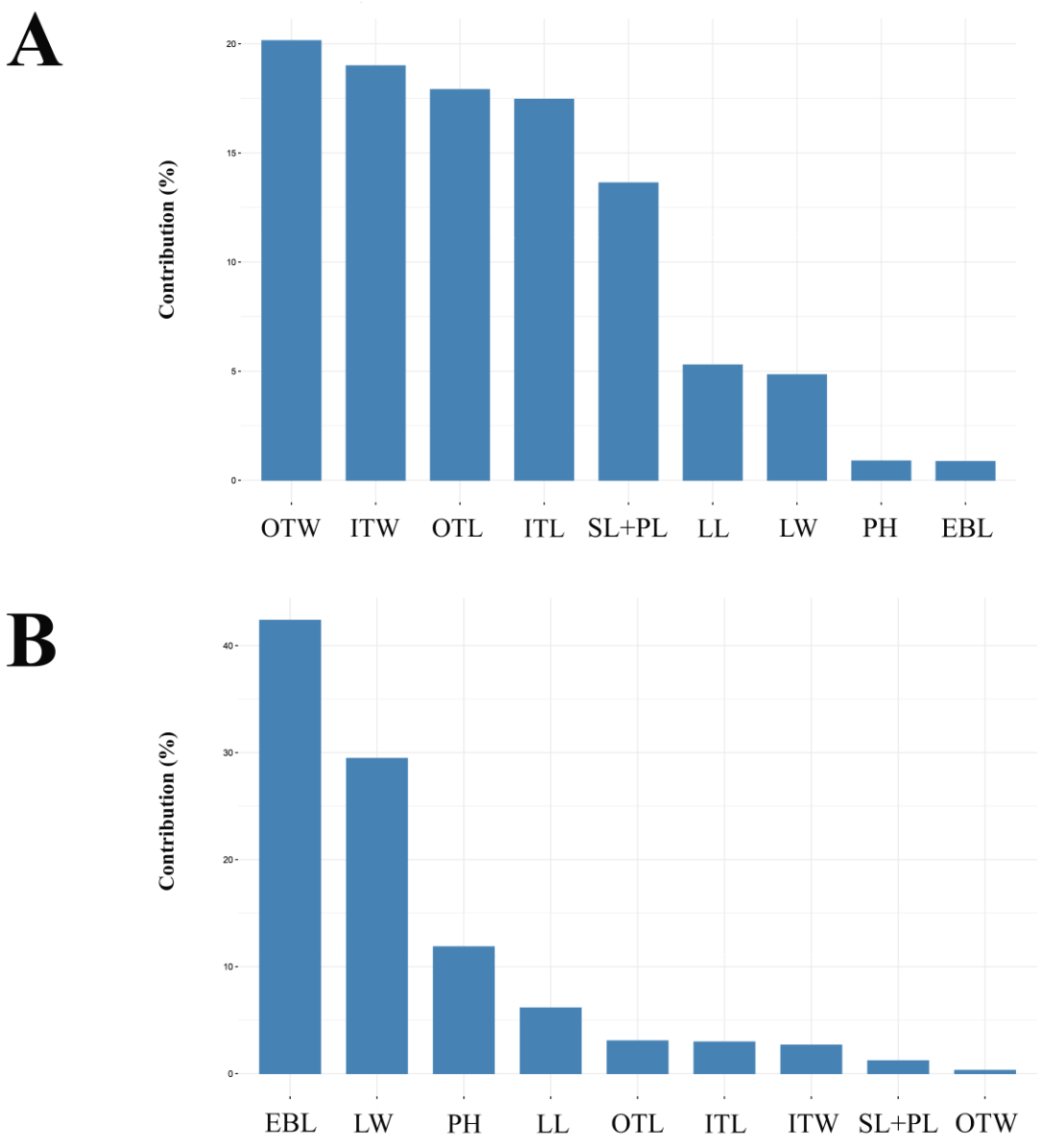

Figure 12. Bar plots of the percentage contribution of each of the nine characters to **A)** principal component 1 and **B)** principal component 2. OTL = outer tepal length; OTW = outer tepal width; IPL = inner tepal length; IPW = inner tepal width; LL = leaf lamina length; LW = leaf lamina width; PH = plant height; SL+PL = scape plus pedicel length; EBL= epigynous beak length.

Seed surface micromorphology

The seed shape of all taxa studied were found to be similar (Figures 13 and 14) - all seeds being ovoid to almost spherical in shape. The diameters of the seeds varied slightly between the five species and putative hybrids. Seeds of *R. deflexa* were longest on average (1.12 –1.24 mm long and 0.71–0.85 mm wide), while those of *R. incompta* were the widest (1.01–1.14 mm long and 0.95–0.99 mm wide). The smallest seeds belonged to *R. thodiana* (0.64–0.71 mm long and 0.50–0.61 mm wide). Seed coat epidermal cells of all five species and putative hybrids are isodiametric, 4–8-gonal with straight, channelled, anticlinal boundaries. Anticlinal cell boundaries were always smooth and well-defined. Overlap in micropapillae was rare in the seeds of all taxa except for *R. rubella*. Considerable variation between the seeds of the five high altitude *Rhodohypoxis* species was found in terms of the type of seed epidermal cells and the distribution pattern of micropappilae of different lengths. These two characters only overlapped between *R. thodiana* and the putative hybrids. These findings are summarized in Table 4.

Table 4. Differences between the seeds of *R. baurii* var. *confecta*, *R. deflexa, R. rubella, R. incompta, R. thodiana* and *putative R. baurii* var. *confecta* × *R. rubella* hybrids (C×R).

Seed characters	***R. baurii* v. *confecta***	***R. deflexa***	***R. rubella***	***R. incompta***	***R. thodiana***	**C×R 6**	**C×R 5**
Size (L×W) in mm	1.01–1.10 ×0.69–0.78	1.12 –1.24 ×0.71–0.85	0.99–1.06 ×0.80–0.86	1.01–1.14 ×0.95–0.99	0.64–0.71 ×0.50–0.61	0.85–0.95 ×0.64–0.83	0.89–0.92 ×0.75–0.91
Types of epidermal cells	colliculate	prismatic flat ocellate and/or colliculate	capitate/pilate	columellate and/or capitate/pilate	colliculate and/or columellate	colliculate and/or columellate	colliculate and/or columellate
Distribution pattern of micropapillae of different lengths	no clear pattern in distribution of different lengths across periclinal wall	very short/absent at centre of epidermal cell/top of convex projection. Gets longer towards anticlinal Boundaries.	shorter at top of convex projection, getting long across periclinal wall and again getting shorter towards anticlinal boundaries	absent/ short at top of convex projection, getting longer across periclinal wall and again getting shorter /absent towards anticlinal boundaries	short at top of convex projection, getting longer across periclinal wall and again getting shorter /absent towards anticlinal boundaries	short at top of convex projection, getting longer across periclinal wall and again getting shorter /absent towards anticlinal boundaries	short at top of convex projection, getting longer across periclinal wall and again getting shorter /absent towards anticlinal boundaries
Micropapillae overlap	rare	rare	common	rare	rare	rare	rare

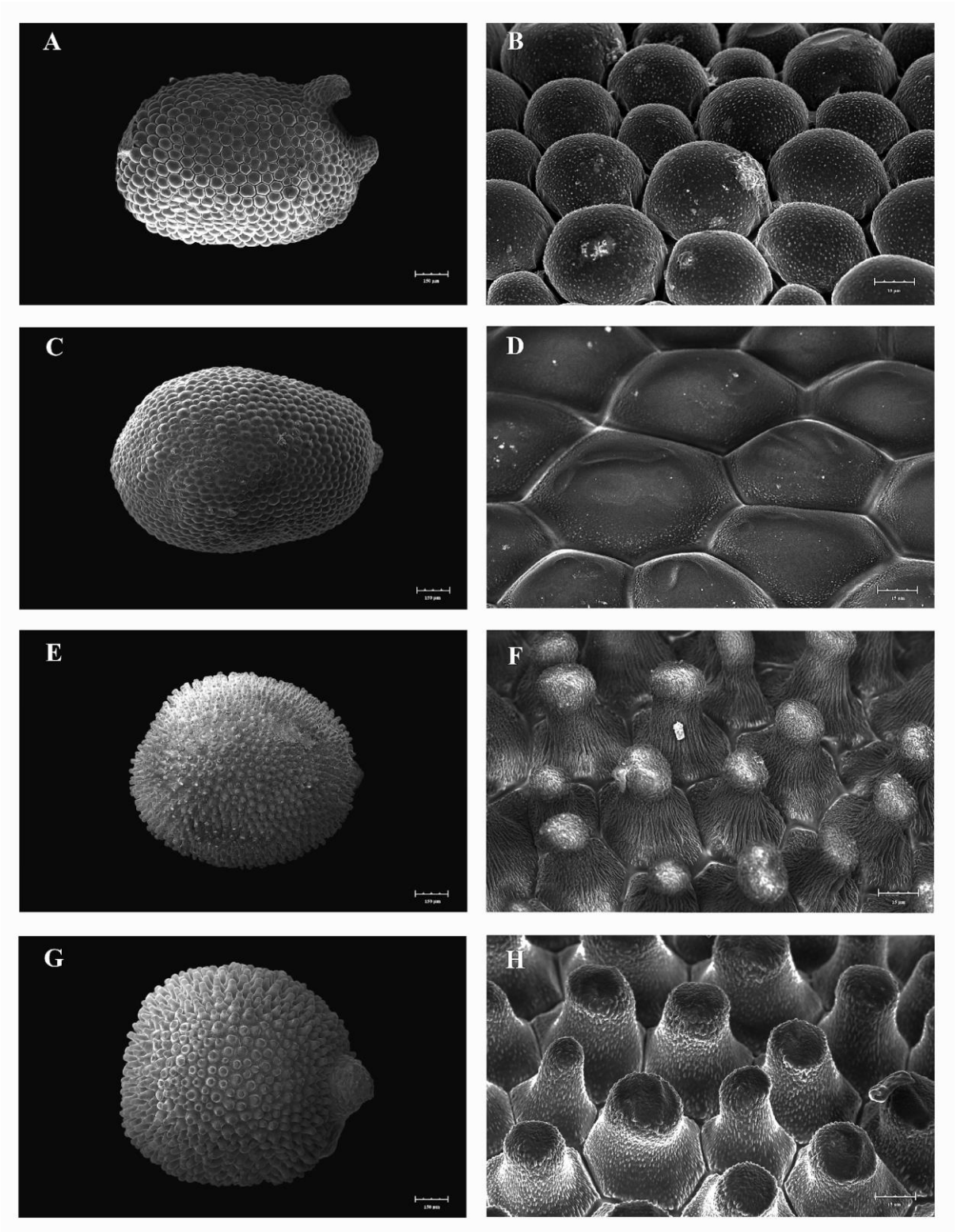

Figure 13. SEM images showing *Rhodohypoxis* seed surface morphology. **(A, B)** *R. baurii* var. *confecta*. **(C, D)** *R. deflexa*. **(E, F)** *R. rubella.* **(G, H)** *R. incompta.* Scale bars of seed images **(A, C, E, G)** = 100 μm; scale bars for seed surface micro-structure images **(B, D, F, H)** = 10 μm.

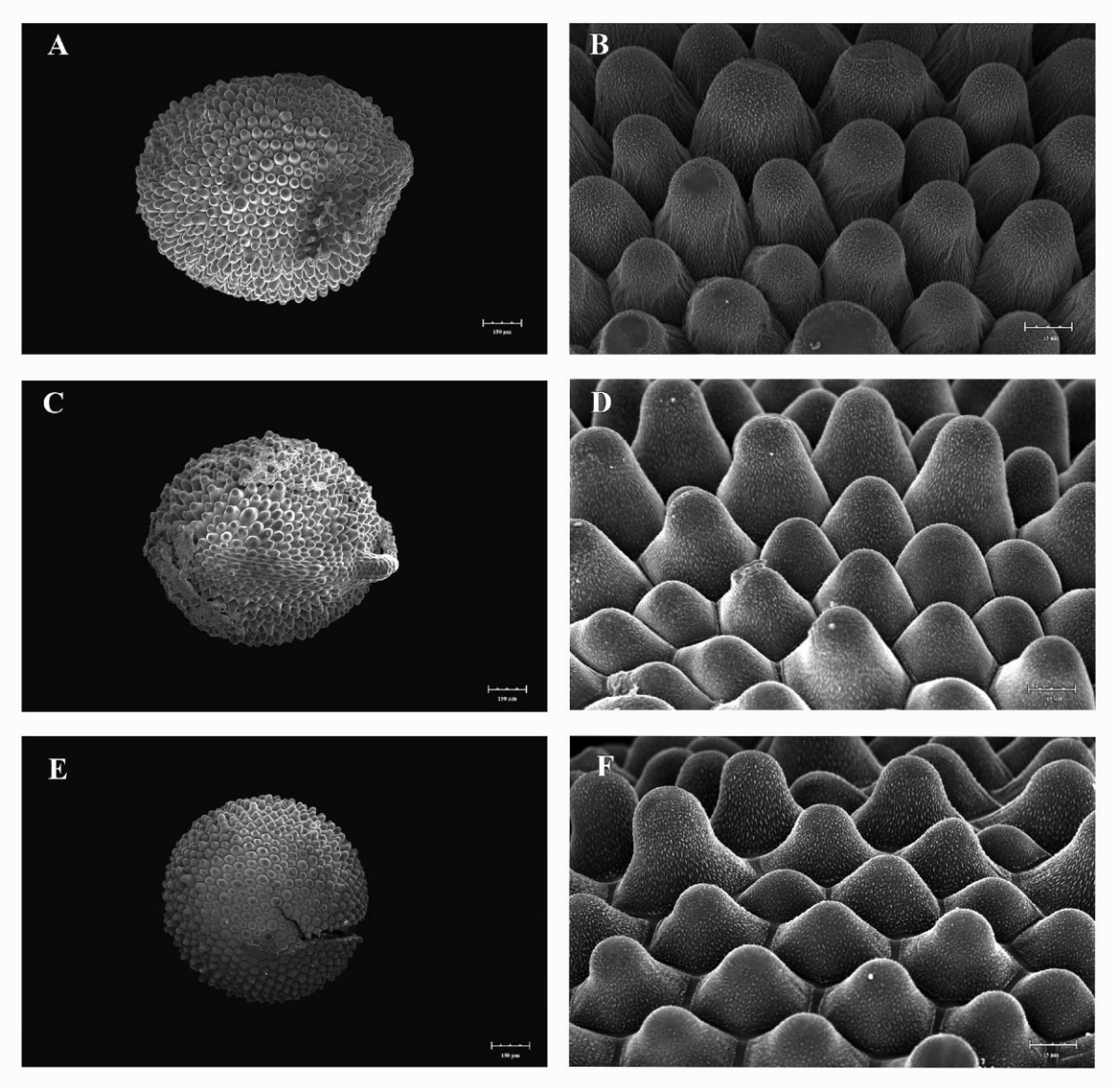

Figure 14. SEM images showing *Rhodohypoxis* seed surface morphology. **(A, B)** *R. thodiana*. **(C, D, E, F)** Putative *R. baurii* var. *confecta* × *R. rubella* hybrids (C×R). Scale bars of seed images **(A, C, E)** = 100 μm; scale bars for seed surface microstructure images **(B, D, F)** = 10 μm.

GENETIC DIFFERENTIATION AND GENE FLOW

Genetic structure across study sites

All loci were polymorphic across samples collected from the three localities (Sentinel Peak, Langalibalele Pass, and near Sani Top). The observed allelic richness (A_o) ranged from 25.167 to 28.167, while the expected allelic richness (A_e) varied between 3.549 and 5.416 across the localities. Additionally, observed heterozygosity (H_o) values ranged from 0.549 to 0.738, while expected heterozygosity (H_s) values spanned from 0.746 to 0.827. The inbreeding coefficient (G_{is}) suggested that there is strong evidence for inbreeding at Langalibalele Pass relative to Sentinel Peak and near Sani Top study sites.

Table 5. Genetic diversity based on six microsatellite markers across three localities. N_{ind} = number of individuals, A_o = the observed number of alleles, A_e = effective number of alleles, H_o = observed individual heterozygosity, Hs = heterozygosity in population, also known as gene diversity, G_{is} = inbreeding coefficient.

Locality	N_{ind}	A_o	A_e	H_o	H_s	G_{is}
Sentinel Peak	55	28.167	5.152	0.705	0.827	0.148
Langalibalele Pass	65	26.667	5.416	0.738	0.824	0.928
near Sani Top	94	25.167	3.549	0.549	0.746	0.264

STRUCTURE analyses were conducted for each study site separately to assess whether hybridization among co-occurring taxa could be detected. For Sentinel Peak, three genetic clusters were identified as optimal by the CLUMPAK software (Kopelman et al., 2015; deltaK =3; see Figure 15A). All three species present at this population (*R. rubella, H. parvula* var. *parvula,* and *R. baurii* var. *confecta*) showed low levels of admixture – with only five individuals having admixture levels ≥10% (RS10, PS12, CS2, CS11, CS22). Of these five, only one (RS10) displayed intermediacy and was correctly assigned as *R. baurii* var. *confecta* × *R. rubella*. The rest were morphologically assigned as either *H. parvula* var. *parvula* (PS12; but was admixed with primarily *R. rubella*, but also *R. baurii* var. *confecta*) or *R. baurii* var. *confecta* (but was admixed with either *H. parvula* var. *parvula* [CS2] or *R. rubella* [CS11, CS22]). Two individuals (RS1 and RS4) were morphologically assigned as *R. rubella* but had pure *R. baurii* var. *confecta* genetic backgrounds. On careful re-examination of these individuals, it was found that they were, in fact, morphologically *R. baurii* var. *confecta* – but were mistaken for *R. rubella* on account of having particularly thin leaves.

From the DAPC for the Sentinel population (Figure 16A), each species forms separate clusters with the *R. baurii* var. *confecta* and *R. rubella* clusters being closer to one another than they are to the *H. parvula* var. *parvula* cluster.

The STRUCTURE results for the Langalibalele Pass locality also indicated that three genetic clusters were represented in the dataset based on the CLUMPAK software (deltaK = 3; see Figure15B). Here, individuals assigned as *H. parvula* var. *parvula* showed very low levels of admixture with none having admixture levels ≥10%. Similarly, only two of the individuals assigned as *R. thodiana* (TL1 and TL19) had admixture levels ≥10%. The one, TL1, was admixed with *R. baurii* var. *confecta*, while the other, TL19, was admixed with both *R. baurii* var. *confecta* and *H. parvula* var. *parvula*. Finally, six of the *R. baurii* var. *confecta* assigned individuals (CL2, CL6, CL7, CL9, CL14, and CL15) had admixture levels ≥10%. They showed evidence of admixture with *R. thodiana* (CL2 and CL15), *H. parvula* var. *parvula* (CL6 and CL9), or both (CL7 and CL14). Of the 18 individuals designated as putative *R. baurii* var. *confecta* × *R. thodiana* hybrids, 10 indeed displayed admixture levels ≥10% for either of these two putative parent taxa (HL1, HL3, HL5, HL6, HL8, HL12, HL13, HL16, HL17, and HL18), while three had a predominantly *R. baurii* var. *confecta* genetic background (HL4, HL9, and HL11), and five had a predominantly *R. thodiana* genetic background (HL2, HL7, HL10, HL14, and HL15). From the DAPC for the Langalibalele population (Figure 16B), each taxon again formed a separate cluster. While *H. parvula* var. *parvula* and *R. thodiana* clusters never overlap with any other cluster, the *R. baurii* var. *confecta* and the putative hybrid clusters do overlap.

Lastly, four genetic clusters were identified by the CLUMPAK software as optimal for the near Sani Top locality (deltaK = 4; see Figure15C). Here, individuals morphologically assigned as *R. deflexa* showed very low levels of admixture with none having admixture levels ≥10%. Five of the individuals morphologically assigned as *R. baurii* var. *confecta* (CT10, CT12, CT16, CT17, and CT18) had admixture levels ≥10%. Three of these (CT10, CT12, and CT18) were primarily admixed with *R. incompta*, and two (CT16 and CT17) with *R. deflexa*. Similarly, five of the individuals morphologically assigned as *R. rubella* (RT1, RT2, RT10, RT19, and RT22) had admixture levels ≥10%. Three showed evidence of admixture with *R. incompta* (RT1, RT2, and RT10), one with *R. baurii* var. *confecta* (RT19), and one with *R. deflexa* (RT22). Seven of the individuals assigned as *R. incompta* (IT1, IT5, IT6, IT7, IT9, IT10, and IT11) had admixture levels ≥10%. Five showed evidence of cryptic admixture with *R. rubella* (IT1, IT5, IT6, IT9, and IT11), one with *R. baurii* var. *confecta*

(IT7), and one with both *R. baurii* var. *confecta* and *R. deflexa* (IT10). One individual (IT4) was assigned as *R. incompta*, but had a near pure *R. rubella* genetic background. This is consistent with the morphological overlap reported in Hilliard & Burtt (1978) between these two taxa, which likely resulted in a misidentification of this *R. rubella* individual – likely because it was at the larger end of the size range for this taxon. The putative *R. baurii* var. *confecta* × *R. deflexa* hybrid (HT1) had a near pure *R. thodiana* genetic background. Of the five putative *R. baurii* var. *confecta* × *R. rubella*/*R. incompta* hybrids (HT2, HT3, HT4, HT5, and HT6), three had a near pure *R. incompta* genetic backgrounds (HT2, HT3, and HT4), one individual (HT5) indeed showed substantial admixture between *R. baurii* var. *confecta* (~15% of the genetic background) and *R. incompta* (~85% of the genetic background), and one (HT6) showed admixture between *R. rubella* and *R. incompta*. From the DAPC for the near Sani Top population (Figure 16C), each taxon again formed a separate cluster. While the *R. deflexa* and *R. baurii* var. *confecta* clusters never overlap with any other cluster, there is some interaction between the *R. rubella* cluster and the putative hybrid cluster. There is also substantial overlap between the *R. incompta* and the putative hybrid cluster.

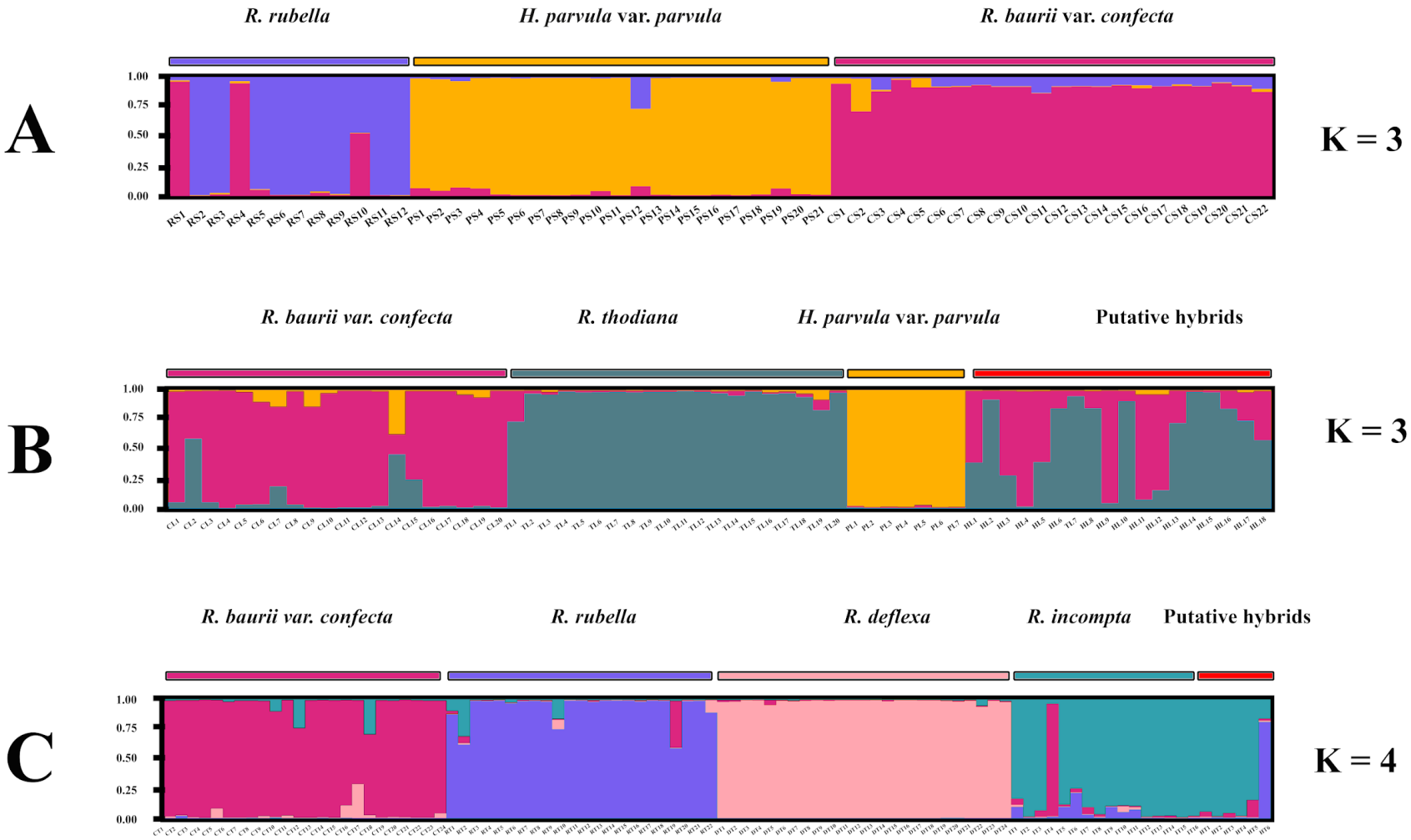

Figure 15. Analysis of the three study sites using STRUCTURE software on 214 individuals, showing genetic affinity among the studied genotypes, grouped to the optimum deltaK. **A**) = Sentinel Peak, **B)** = Langalibalele Pass, **C)** = near Sani Top.

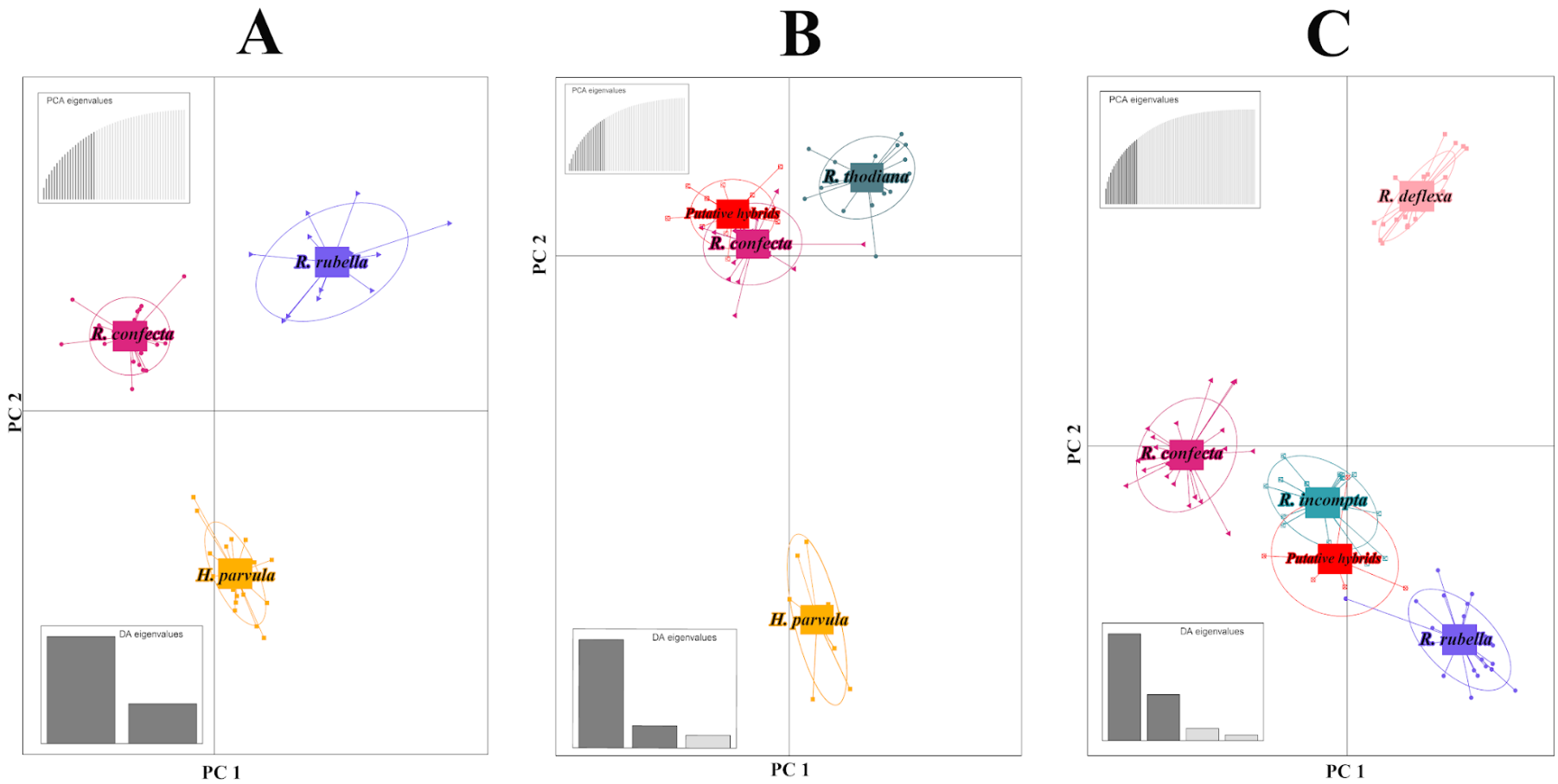

Figure 16. Scatter plots of the discriminant analysis of principal components (DAPC) of the microsatellite data from three separate study sites. The axes represent the first two linear discriminants (LD). Each circle represents a cluster, and each shape represents an individual. Eigenvalues of the analysis are displayed in the inset. **A)** = Sentinel Peak, **B)** = Langalibalele Pass, **C)** =. near Sani Top.

Genetic structure across species

The genetic diversity of the six species varied considerably. *Rhodohypoxis baurii* var. *confecta* displayed the highest observed number of alleles (A_o = 19.500) and effective number of alleles (A_e = 8.547), while *R. deflexa* exhibited the lowest observed number of alleles (A_o = 6.667) and effective number of alleles (A_e = 4.121), suggesting reduced genetic diversity. The observed individual heterozygosity (H_o) ranged from 0.433 in *R. deflexa* to 0.825 in *H. parvula* var. *parvula* reflecting variations in genetic variability between the studied species. Heterozygosity in the population (H_s) ranged from 0.718 in *R. rubella* to 0.928 in *H. parvula* var. *parvula*. The inbreeding coefficient (Gis) was low across all species.

Table 6. Genetic diversity based on six microsatellite markers across six species. N_{ind} = number of individuals, A_o = the observed number of alleles, A_e = effective number of alleles, H_o = observed individual heterozygosity, H_s = heterozygosity in population, also known as gene diversity, G_{is} = inbreeding coefficient.

Species	N_{ind}	A_o	A_e	H_o	H_s	G_{is}
R. baurii var. *confecta*	42	19.500	8.547	0.703	0.870	0.191
R. rubella	35	12.333	4.222	0.528	0.718	0.265
R. thodiana	20	12.000	6.702	0.815	0.816	0.001
R. deflexa	25	6.667	4.121	0.433	0.741	0.415
R. incompta	16	8.500	5.061	0.457	0.829	0.448
H. parvula var. *parvula*	28	18.000	11.354	0.825	0.928	0.111

The pairwise species F_{ST} values visualized using a heatmap (Figure 17) offers insights into the genetic differentiation among the six species (*R. rubella, H. parvula* var. *parvula, R. baurii* var. *confecta, R. thodiana, R. deflexa,* and *R. incompta*). F_{ST} values ranged from 0.068 to 0.257 across species pairs, indicating varying degrees of genetic divergence. The highest pairwise F_{ST} value of 0.257 was observed between *R. deflexa* and *R. thodiana*, suggesting significant genetic differentiation between these two species. On the other hand, *R. rubella* and *R. incompta* exhibited the lowest pairwise F_{ST} value, suggesting they have the closeted genetic relationship among the species examined.

From the DAPC across all species (excluding putative hybrids; Figure 18), each taxon again forms a separate cluster. While the *R. deflexa* cluster never overlaps with any other cluster, there is substantial overlap between the *R. rubella* and *R. incompta* clusters, the *R. baurii* var. *confecta* and the *H. parvula* var. *parvula* clusters, and finally the *R. baurii* var. *confecta* and the *R. thodiana* clusters.

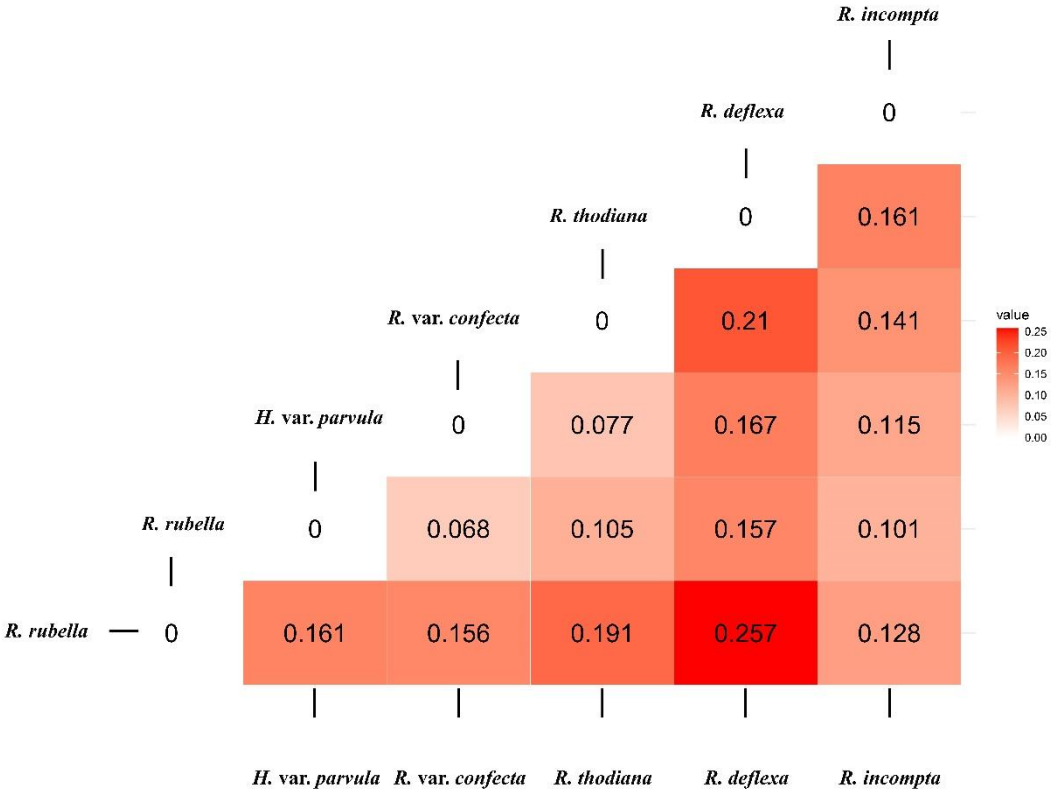

Figure 17. F_{ST} heatmap of the six species (*R. rubella*, *H. parvula* var. *parvula*, *R. baurii* var. *confecta*, *R. thodiana*, *R. deflexa*, and *R. incompta*).

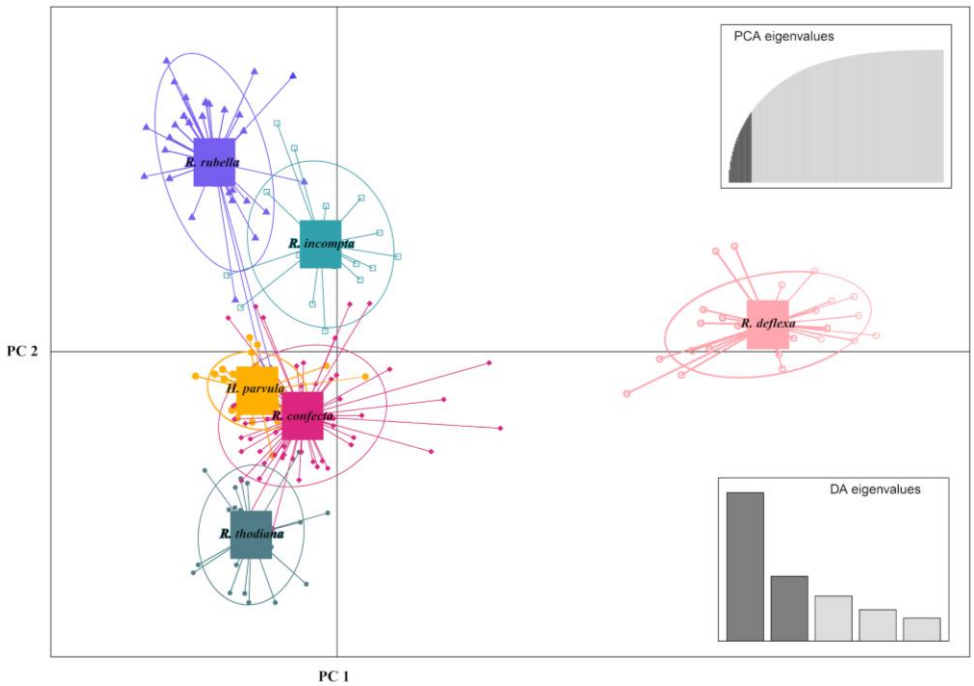

Figure 18. Scatter plots of the discriminant analysis of principal components of the microsatellite data of all six species (*R. rubella*, *H. parvula* var. *parvula*, *R. baurii* var. *confecta*, *R. thodiana*, *R. deflexa*, and *R. incompta*).

CROSSING EXPERIMENT

Flower phenology overlap

Hypoxis parvula var. *parvula* typically flowers from early November to the end of March, while *R. baurii* var. *confecta* flowers from September to February. The flowers of *H. parvula* var. *parvula* are nyctinastic, while *R. baurii* var. *confecta* flowers are not (D. Coetzer, personal observation). Although technically once mature, *R. baurii* var. *confecta* flowers remain "open" irrespective of the time of day or weather conditions, its stigma, style, and the stamens are always hidden due to its floral morphology. For a list of voucher specimens used in the analysis of flowering time overlap, refer to the Appendix for the complete list.

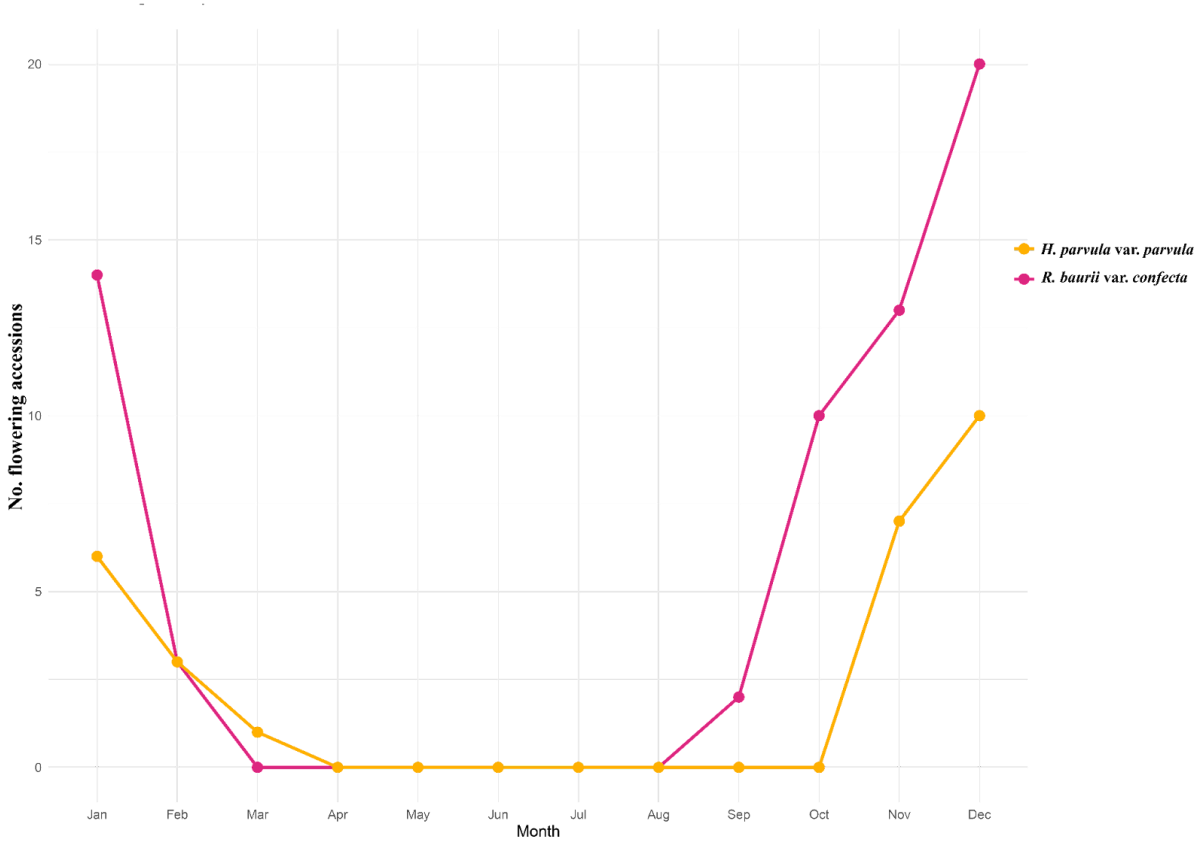

Figure 19. Line plots depicting the flower phenology of *H. parvula* var. *parvula* and *R. baurii* var. *confecta*.

Pollination and seed-set

The study involved a total of 203 hand pollinations and self-pollinations, with an average of 33 hand pollinations per pollination-type, (ranging from 25 to 48). These pollinations resulted in a total of 509 seeds (Table 7). All the *H. parvula* var. *parvula* plants used in the study demonstrated 100% self-incompatibility, as none of the flowers that were self-pollinated set seed. These *H. parvula* var. *parvula* plants only produced seeds when they were crossed with each other (52% outcrossed flowers set seed) or when used as the paternal donor in crosses with *R. baurii* var. *confecta* maternal plants (48% outcrossed flowers set seed).

In contrast, *R. baurii* var. *confecta* plants exhibited a mixed reproductive strategy, demonstrating both self-pollination (48% self-pollinated flowers set seed) and outcrossing ability (32% outcrossed flowers set seed). However, when *R. baurii* var. *confecta* plants were used as pollen donors on *H. parvula* var. *parvula* maternal plants, no seeds were produced. Furthermore, none of the five emasculated *R. baurii* var. *confecta* and five emasculated *H. parvula* var. *parvula* individuals showed any signs of apomictic breeding, as they did not set seed.

Table 7. Total number of seeds produced per pollination-type performed during the 2022 flowering season.

Poll. type	No. of hand pollinations	Seeds	Mean	SE	SD
P-s	25	0	0	0	0
C-s	48	243	5.1	8.04	1.16
C♀ × C♂	37	54	1.4	0.52	3.18
P♀ × P♂	25	111	7.5	1.94	9.70
P ♀ × C♂	31	0	0	0	0
C ♀× P♂	37	112	3.0	1.02	6.26

Pollination type refers to P-s = *H. parvula* var. *parvula* self-pollinated, C-s = *R. baurii* var. *confecta* self-pollinated, C × C = *R. baurii* var. *confecta* crossed with *R. baurii* var. *confecta,* P × P = *H. parvula* var. *parvula* crossed with *H. parvula* var. *parvula*, P♀ × C ♂ = *H. parvula var. parvula* pollinated with *R. baurii* var. *confecta,* C♀ × P ♂ = *R. baurii* var. *confecta* pollinated with *H. parvula var. parvula.* No. of crosses = Total number of pollinations performed, Seeds = Total number of seeds produced, mean = Total mean number of seeds produced of each pollination type. SE = standard error, SD = standard deviation. ♀ = seed parent; ♂ = pollen parent.

A Kruskal-Wallis rank sum test was used to assess the potential differences in seed set across the different pollination-types. The results showed a statistically significant difference in seed set among the six pollination-types (Kruskal-Wallis chi-squared = 40.863, df = 5, P < 0.001). These findings indicate that pollination-types have a significant impact on the seed set, with statistically significant differences observed among the different pollinations performed.

To further explore the specific pollination differences, pairwise comparisons were performed using Conover's Test with a Bonferroni correction. I found that there was no significant difference between groups *R. baurii* var. *confecta* pollinated with *H. parvula* var. *parvula* and *H. parvula* var. *parvula* pollinated with *R. baurii* var. *confecta*. There was a significant difference between groups *H. parvula* var. *parvula* pollinated with *R. baurii* var. *confecta* and *R. baurii* var. *confecta* self-pollinated, as well as between groups *R. baurii* var. *confecta* pollinated with *H. parvula* var. *parvula* and *H. parvula* var. *parvula* cross pollinated with *H. parvula* var. *parvula*. There is a significant difference between *H. parvula* var. *parvula* cross pollinated with *H. parvula* var. *parvula* and *H. parvula* var. *parvula* self-pollinated.

Table 8. Results from the pairwise comparisons of cross types using Conover's test with a Bonferroni correction.

Comparison	Mean Difference	p-value	Significant at α=0.05
C×P vs C×C	-1.650466	0.7533	No
C×S vs C×C	-2.069227	0.2987	No
C×S vs C×P	-0.315214	0.2987	No
P×C vs C×C	2.714101	1.0000	No
P×C vs C×C	2.714101	0.0543	No
P×C vs C×P	4.290071	0.0002*	Yes
P×C vs C×S	4.832695	0.0000*	Yes
P×P vs C×C	-2.614444	0.0722	No
P×P vs C×P	-1.132282	1.0000	No
P×P vs P×C	-4.976449	0.0000*	Yes
P×S vs C×C	2.552547	0.0859	No
P×S vs C×P	4.034709	0.0006*	Yes
P×S vs C×S	4.514718	0.0001*	Yes
P×S vs P×C	0.000000	1.0000	No
P×S vs P×P	4.729527	0.0000*	Yes

Pollination type refers to P×S = *H. parvula* var. *parvula* self-pollinated, C×S = *R. baurii* var. *confecta* self-pollinated, C×C = *R. baurii* var. *confecta* crossed with *R. baurii* var. *confecta*, P×P = *H. parvula* var. *parvula* crossed with *H. parvula* var. *parvula*, P×C = *H. parvula* var. *parvula* pollinated with *R. baurii* var. *confecta*, C×P = *R. baurii* var. *confecta* pollinated with *H. parvula* var. *parvula*..The first parent mentioned in a cross is always the seed parent, while the second is the pollen parent.

Seed germination

About 51.7 % of all seeds produced were used in the germination trail. The germination data revealed that 50% resulting from the *R. baurii* var. *confecta* × *R. baurii* var. *confecta* crosses germinated (Table 9). I also found that 48.8% of the seeds from *R. baurii* var. *confecta* self-pollinations germinated, which is similar to the 46.6% germination success for seeds from the *H. parvula* var. *parvula* × *H. parvula* var. *parvula* pollinations. It is noteworthy that only 8.5% of seeds from the *R. baurii* var. *confecta* × *H. parvula* var. *parvula* pollinations germinated.

Table 9. Total number of seeds successfully germinated per cross-type planted of the seeds produced during the 2022 flowering season.

Cross type	No. planted	Germ.	Germ. %	SE	SD
C-s	88	43	48.86	2.92	7.16
C × C	34	17	50.00	1.55	3.81
P × P	88	41	46.59	1.72	5.17
C × P	59	5	8.47	1.01	0.33

Pollination type refers to C-s = *R. baurii* var. *confecta* self-pollinated, C × C = *R. baurii* var. *confecta* crossed with *R. baurii* var. *confecta,* P × P = *H. parvula* var. *parvula* crossed with *H. parvula* var. *parvula, C* × P = *R. baurii* var. *confecta* pollinated with *H. parvula var. parvula.* Germ. = Total number of seeds germinated, Germ. % = successful germination percentage, SE = standard error, SD = standard deviation. The first parent mentioned in a cross is always the seed parent, while the second is the pollen parent.

Based on the generalised linear model (GLM) conducted on germination success (Table 10), there were differences among pollination types that produced seed and their effect on germination success. The *R. baurii* var. *confecta* × *H. parvula* var. *parvula* cross exhibited a significantly lower germination success compared to the mean (Z-value = -4.364; P-value < 0.0001). None of the other cross types exhibited a significant effect on germination success.

Table 10. Results from GLM conducted on the effect of cross type on germination success. P-values were considered significant when they were ≤ 0.05.

Cross type	Estimate	Z-value	P-value
P × P	-0.13658	-0.639	0.523
C × P	-2.24297	-4.364	0.0001
C × C	0.13658	0.338	0.735
Cs	0.09111	0.302	0.763

Estimation of DNA index

Rhodohypoxis baurii var. confecta (n =15) and *H. parvula var. parvula* (n =15) individuals from Sentinel Peak car park used in the experimental crosses and pollen size measurements were also cytotyped via flow cytometry to estimate their DNA index. All *R. baurii var. confecta* were confirmed to have a DNA index of approximately 1.79 – 1.88 and *H. parvula var. parvula* plants were confirmed to be roughly double that amount at approximately 3.85 – 4.02. These findings correspond to previous cytotyping studies conducted at this locality (Niemann et al., 2023, Ferreria et al., in prep). The DNA indices for *R. baurii* var. *confecta* × *H. parvula* var. *parvula* crosses differed from the expectation of an intermediate DNA index between its two parents. All *R. baurii* var. *confecta* × *H. parvula* var. *parvula* F1 plants that germinated successfully had a DNA index of ~1.73 – 1.80 (n = 4).

Table 11. Nuclear DNA content estimations of *R. baurii* var. *confecta* individuals (n = 15) and *H. parvula* var. *parvula* individuals (n = 15) used in the crossing experiments, as well as those of the resulting hybrid *R. baurii* var. *confecta* × *H. parvula* var. *parvula* offspring (n = 4).

Taxon	Sample ID	Standard G0/G1 peak MFI	Standard CV (%)	Sample G0/G1 peak MF	Sample CV (%)	DNA index
R. baurii var. *confecta*	C1	108.65	3.54	195.92	2.41	1.8
	C2	119.62	4.45	214.88	3.1	1.79
	C3	103.85	3.45	188.19	2.28	1.81
	C4	101.66	5.46	186.34	4.32	1.83
	C5	110.95	3.47	202.66	2.55	1.82
	C6	102.18	3.76	185.47	2.24	1.81
	C7	111.12	4.14	204.04	2.83	1.83
	C8	109.36	8.05	200.28	2.92	1.83
	C9	111.42	3.44	202.33	2.39	1.81
	C10	107.83	5.42	200.39	6.53	1.85
	C11	102.23	3.79	187.83	2.89	1.83

	C12	100.97	3.33	182.78	2.24	1.81
	C13	101.78	3.82	185.5	2.1	1.82
	C14	102.48	3.73	185.35	2.14	1.8
	C15	104.88	5.59	189.97	3.38	1.88
H. parvula var. *parvula*	P1	101.48	4	393.61	2.68	3.87
	P2	102.26	4	394.46	2.92	3.85
	P3	100.63	3.88	395.81	2.86	3.93
	P4	101.98	3.66	392.89	2.58	3.85
	P5	101.28	3.7	396.69	2.7	3.92
	P6	100.02	7.12	412.03	4.66	4.12
	P7	96.96	8.26	416.6	4.88	4.03
	P8	99.44	6.53	400.06	4.79	4.02
	P9	96.55	7.99	398.66	3.95	4.13
	P10	97.02	7.34	401.26	4.41	4.13
	P11	98.76	8.16	398.41	4.39	4.03
	P12	99.15	9.33	398.62	5.78	4.02
	P13	95.78	8.43	393.76	5.13	4.11
	P14	102.35	7.99	417.69	4.58	4.08
	P15	95.43	10.6	397.9	5.98	4.17
R. baurii var. *confecta* × *H. parvula* var. *parvula*	H1	102.92	4.77	180.46	3.54	1.75
	H2	100.92	5.71	177.5	3.62	1.75
	H3	241.16	4.55	419.54	1.91	1.73
	H4	101.14	2.86	182.79	1.87	1.8

Pollen analyses

The pollen viability was assessed for the fifteen *H. parvula* var. *parvula* and fifteen *R. baurii* var. *confecta* individuals from Sentinel Peak car park that were included in the crossing experiment (Figure 20; see Table A2 for pollen viability data for each specimen). The results of the Welch Two Sample t-test indicated that there was no significant difference between the pollen viability of these two species (t = -0.734, df = 21.23, P = 0.471).

A Spearman's rank correlation test was conducted to investigate the relationship between DNA index levels and viable pollen size in the *R. baurii* var. *confecta* (C1- C15) from Sentinel Peak car park compared to *R. baurii* var. *platypetala* (M1- M3) from Minerva Private Nature Reserve (see Table A3 for pollen size summary statistics). The analysis revealed a statistically significant positive correlation between DNA index levels and mean pollen size (Spearman's rho = 0.975, P = < 0.00001^6). This indicates that as the DNA index level increases, there is a trend for pollen size to increase. These findings suggest that DNA index may play a role in determining mean pollen size in *R. baurii. var. confecta.*

A Spearman's rank correlation test was also conducted to investigate the relationship between DNA index levels and viable pollen size in *H. parvula* var. *parvula* (P1-P15) from Sentinel Peak car park compared to *H. parvula var. parvula* (PD1- PD3) from Monk's Cowl. The analysis revealed that there was also a moderate positive correlation observed between the mean viable pollen size values and DNA index of the sampled *H. parvula* var. *parvula* individuals (Spearman's rho = 0.647, P = 0.003). This suggests that as the mean pollen size of the samples increases, there is likely to be a corresponding increase in the DNA index. This finding provides insights into the relationship between these two variables and may indicate potential associations between DNA index and mean pollen size. Notably, there were three outliers (P3, P8, P1) in the *H. parvula* var. *parvula* pollen size samples from Sentinel Peak that showed the pollen size of a much lower DNA-index sampled sizes.

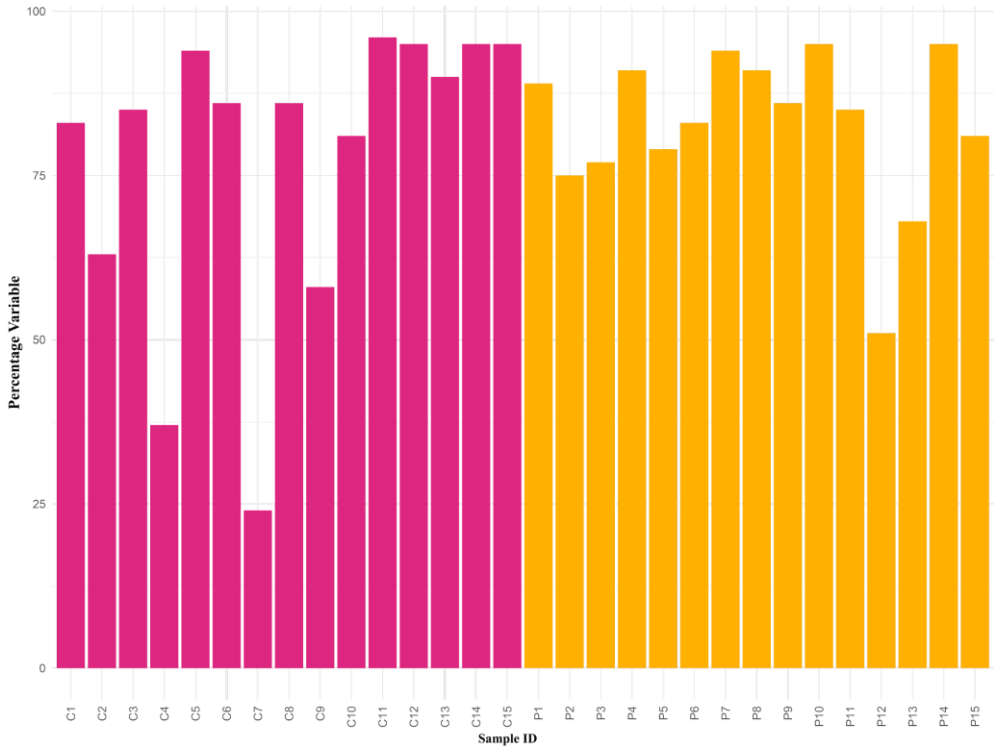

Figure 20. The pollen viability data for *R. baurii* var. *confecta* (C1-C15) and *H. parvula* var. *parvula* (P1-P15) from Sentinel Peak.

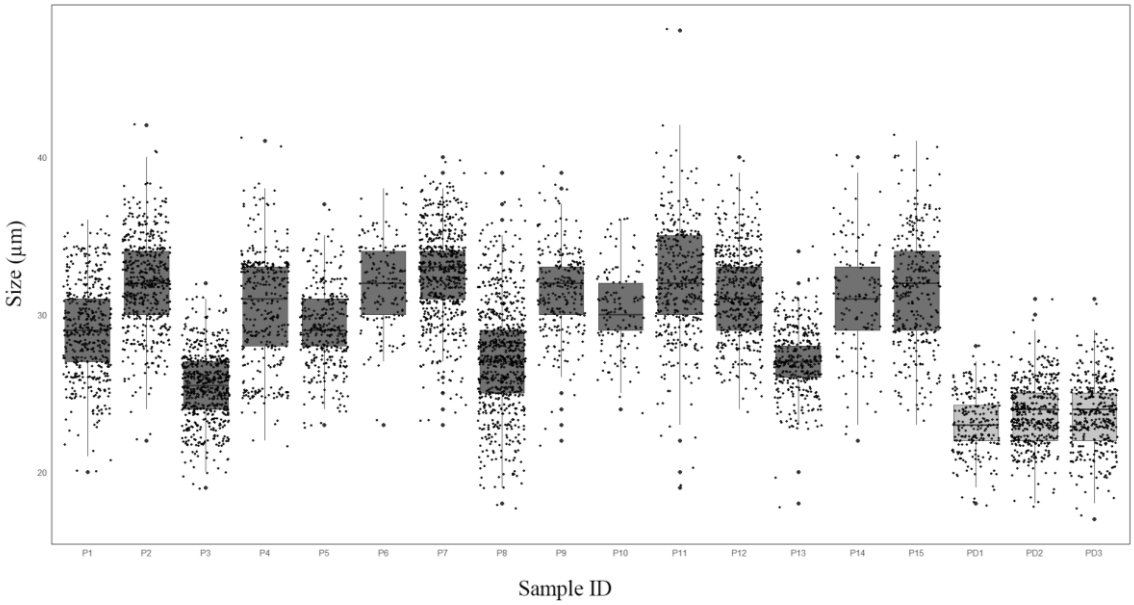

Figure 21. A box plot comparison of pollen sizes (mean and standard error bars) for *R. baurii* var. *confecta* (C1-C15) from Sentinel Peak, Free State, and *R. baurii* var. *platypetala* (M1-M3) from Minerva Nature Reserve, Kwa-Zulu Natal, South Africa.

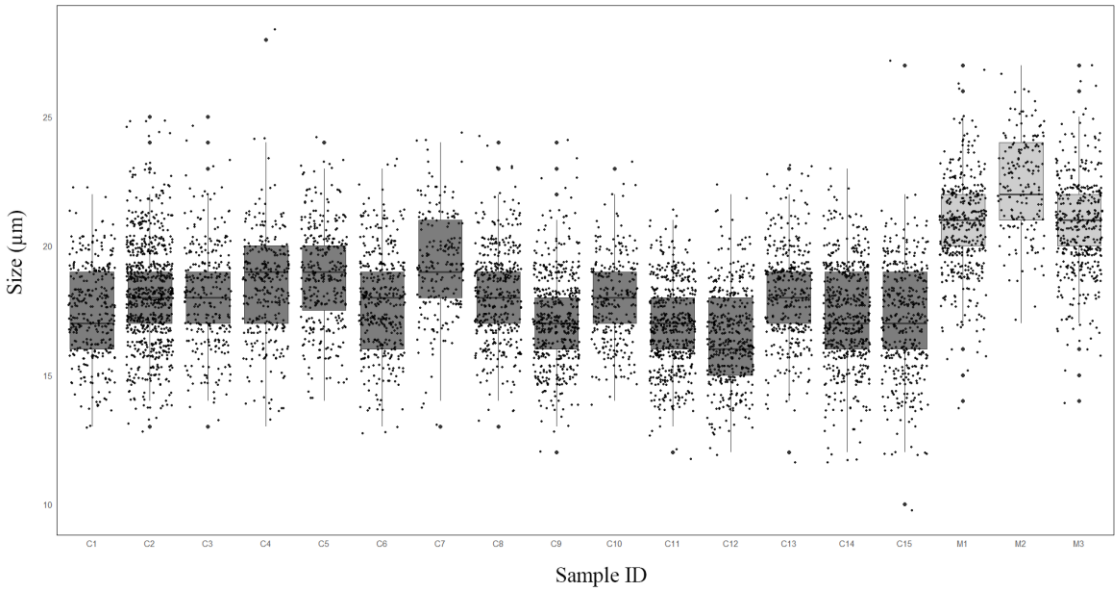

Figure 22. The pollen size data for *H. parvula* var. *parvula* (P1-P15) from Sentinel Peak carpark and *H. parvula* var. *parvula* (PD1-PD3) from Monks Cowl Nature Reserve.

CHAPTER 4: DISCUSSION

Published accounts based on morphological traits (Hilliard & Burtt, 1978; Singh, 2009) suggested that the high altitude *Rhodohypoxis* species (*R. baurii* var. *confecta*, *R. rubella*, *R. deflexa*, *R. incompta* and *R. thodiana*) and *H. parvula* var. *parvula* hybridize in areas where they grow in sympatry in the Drakensberg Alpine Centre (DAC). These observations led me to use multiple lines of evidence to examine whether hybrids were present at three study sites (near Sani Top, Langalibalele Pass, and Sentinel Peak) where species co-occur to evaluate the discreteness of each species in accordance with the general lineage species concept (GLSC; De Queiroz 2005, 2007), to assess the presence or absence of interspecific/intergeneric gene flow, and to evaluate the contribution of each species to putative hybrid individuals.

Results from the PCA demonstrated that each of the five high-altitude *Rhodohypoxis* species (*R. baurii* var. *confecta*, *R. rubella*, *R. delexa*, *R. incompta*, and *R. thodiana*) could be separated into unique clusters by the morphological characters used. Of these characters, only the leaf lamina length (LL) did not substantially contribute to either of the first two principal components. These findings corroborate the emphasis on floral character differences by Hilliard & Burtt (1978) for delineating *Rhodohypoxis* species. It should be noted that specific leaf characters emphasized by Hilliard & Burtt (1978), i.e. venation and hairiness, were not used here, despite their purported importance as diagnostic characters. Their inclusion in future studies may further refine the results shown here.

The inclusion of putative hybrids into the PCA resulted in an additional cluster that partially overlapped with *R. baurii* var. *confecta*, *R. incompta* and *R. deflexa* and completely overlapped with *R. thodiana*. In their revision, Hilliard & Burtt (1978) documented putative *R. baurii* var. *confecta* × *R. thodiana* hybrids and *R. rubella* × *R. thodiana* hybrids at Langalibalele pass, and putative *R. baurii* var. *confecta* × *R. deflexa* hybrids near Sani top – of which only the putative *R. rubella* × *R. thodiana* hybrids could not be re-located for the current study. Interestingly, no individual resembling *R. rubella* was found at Langalibalele pass in the current study – even after revisiting the pass multiple times over three years. This may be because *R. rubella* is not plentiful at this location and was simply missed or because all individuals collected from this location designated as *R. rubella* have flatter more hairy leaves than usual for this taxon (Hilliard & Burtt, 1978) – and was simply not recognised as *R. rubella*. Alternatively, as was postulated by Hilliard & Burtt (1978), it might be possible

that the whole of the Langalibalele *R. rubella* population has been introgressed by the more numerous *R. thodiana* – potentially to the point of being indistinguishable from the *R. thodiana* at this locality.

Surprisingly, in the PCA, *R. rubella* was the only species that had no overlap with the putative hybrid grouping, despite the putative hybrid individuals found in the field that appeared to have traits that coincided with *R. rubella*. For example, at Sentinel Peak, a putative hybrid individual was found growing in a sympatric *R. baurii* var. *confecta* and *R. rubella* population and was designated as a hybrid between these two species (K. Glennon pers. obs.). This putative *R. baurii* var. *confecta* × *R. rubella* hybrid was a much less hairy plant than *R. baurii* var. *confecta* individuals and had a flower with a very short pedicel (~3–4 mm). This putative hybrid also had a glabrous, colourless, areal ovary that was thin walled, elliptic in shape, ~3–5mm long, had flattened appearance, and a well-developed epigynous beak. Furthermore, its leaves were intermediate in width between its putative parents (0.5–2 mm in *R. rubella*, ~2–3 mm in the putative hybrid, 3–8 mm in *R. baurii* var. *confecta*), lanceolate, keeled, folded, acuminate, stiff, bright green, and very sparsely hairy near margins and over the keel. Prior to visiting the type locality of *R. thodiana*, these Sentinel hybrids were designated as such based on the areal ovary with an epigynous beak, which is diagnostic for *R. thodiana*. Unlike this putative hybrid, however, *R. thodiana* has hairs on its green, elliptic or subrotund, 2–3 mm long ovary. Its leaves are also characteristically pilose (either on both surfaces or only the adaxial surface with a nearly glabrous abaxial surface) and has a conspicuous intramarginal vein.

A putative hybrid individual that appeared to be similar to the putative hybrid individual from Sentinel Peak was collected near Sani Top (see Figure 23) – once again within a sympatric *R. baurii* var. *confecta* and *R. rubella* population. These hybrid plants, however, were more abundant than at Sentinel and had a hairy, green, areal ovary as well as a much longer pedicel (30–45 mm). Due to the presence of *R. incompta* near the Sani population (within a 500 m radius), it is possible that *R. incompta* could also have contributed some of these intermediate traits, therefore these individuals were designated as putative *R. baurii* var. *confecta* × *R. rubella*/*R. incompta* hybrids. *Rhodohypoxis incompta* could feasibly either have contributed directly to this taxon by being a putative parent with *R. baurii* var. *confecta* or indirectly via the potential *R. rubella* × *R. incompta* hybrids present at this location as suggested by Hilliard & Burtt (1978).

Due to the close relationship proposed by Hilliard & Burtt (1978) between *R. rubella* and *R. incompta*, the numerous synapomorphies between them would make a putative *R. rubella* × *R. incompta* hybrid exceptionally challenging to detect morphologically – with the only indication of such a cross postulated to be plants with floral and vegetative characters that would be intermediate in size between its putative parents.

Putative hybrids between *R. baurii* var. *confecta* (with aerial ovaries) and either of the *Rhodohypoxis* taxa with subterranean ovaries (*R. rubella* and *R. incompta*) seem to consistently result in hybrid offspring with intermediate ovaries approximating those of *R. thodiana*. This could explain the complete overlap between the putative hybrids cluster and the *R. thodiana* cluster in the PCA and might suggest that *R. thodiana* may be of hybrid origin – likely between *R. baurii* var. *confecta* and *R. rubella*. Seed surface micromorphological comparisons also supports this hypothesis. Seed surface micromorphology was found to be different for each of the five high-altitude *Rhodohypoxis* species and is, therefore, of substantial taxonomic value within *Rhodohypoxis* – just like it is in *Hypoxis* (Niemann et al., 2023). Analysis of the seed surface micromorphology of putative *R. baurii* var. *confecta* × *R. rubella*/*R. incompta* hybrids, however, revealed that they were nearly indistinguishable from the seeds of *R. thodiana*. Further work needs to be done to validate these observations.

The *Rhodohypoxis baurii* varieties were the target taxa for the development of the microsatellites employed in this study. Producing new microsatellite loci for different species *de novo* is a labour-intensive and costly process, but cross-species amplification of previously developed microsatellites has been used successfully as a more affordable alternative (Mengistu et al., 2020). It is widely known that the evolutionary distance between species has an inverse relationship with the likelihood of a successful interspecific/intergeneric (heterologous) amplification of any DNA sequence (Butcher et al., 2000; Oliveira et al., 2013). The substantial putative hybridization reported between *R. baurii* var. *confecta* and many of the other taxa included in this study (Hilliard & Burtt, 1978; Singh, 2009), together with the close genetic distance inferred between *Hypoxis* and *Rhodohypoxis* in previous phylogenetic studies (Kocyan et al., 2011; Birch & Kocyan, 2021; Niemann et al., 2023) suggested suitable evolutionary distance between these taxa to take advantage of the previously developed microsatellite loci. Indeed, in this study, even when including *H. parvula* var. *parvula*, good transferability was obtained because all the species could be amplified by 100% of the primer pairs used. This transferability represents a potential source

of codominant markers for evolutionary, ecological, and conservation studies across species and even genus boundaries within the Hypoxidaceae. Investigating the genetic distance at which the transferability of these markers' decreases would go a long way in establishing the extent of their utility within this family.

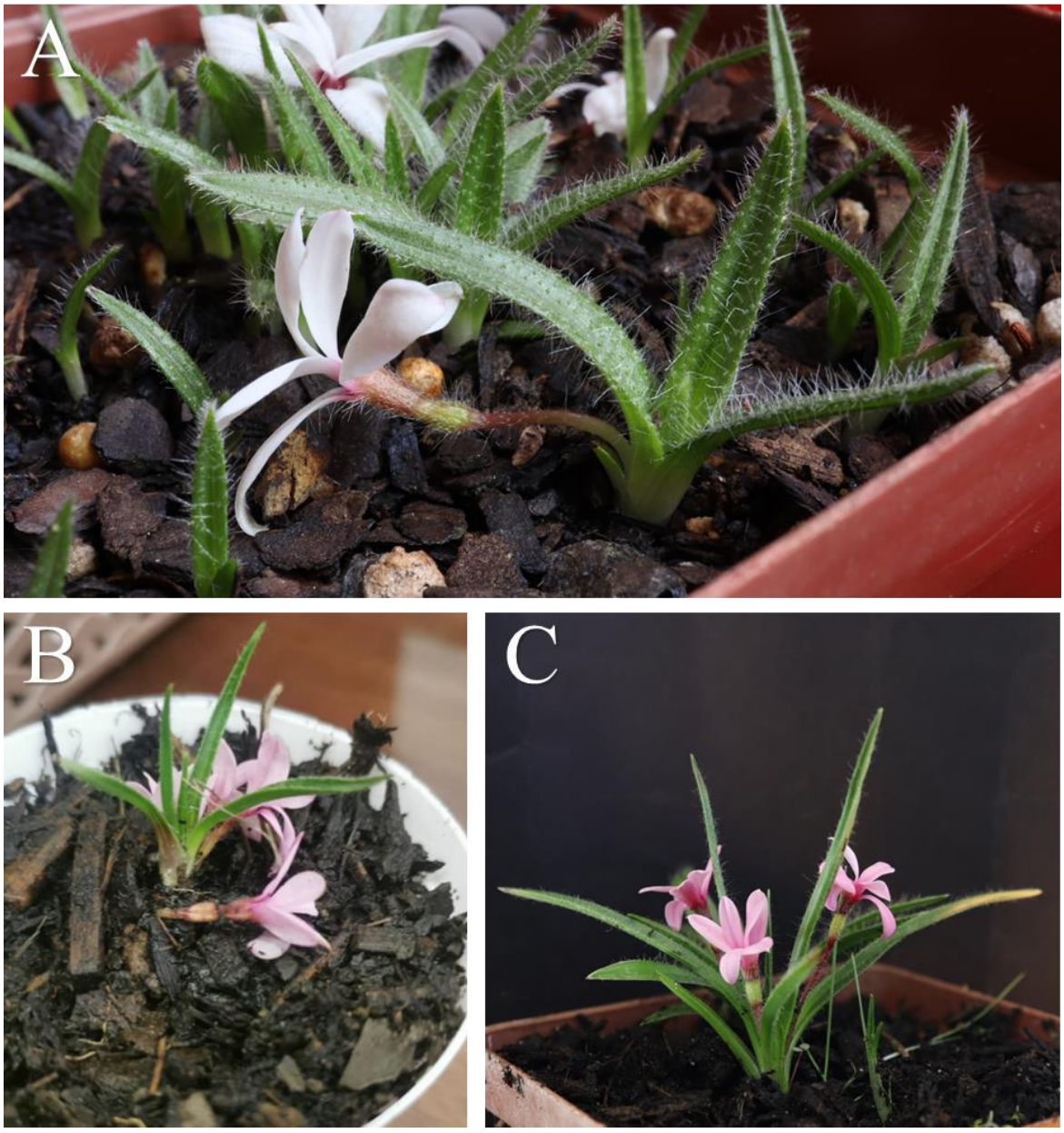

Figure 23. A) Photographs of *R. thodiana* from Langalibalele pass **B)** *R. baurii* var. *confecta* × *R. rubella* from Sentinel peak **C)** *R. baurii* var. *confecta* × *R. rubella*/*R. incompta* from near Sani Top.

The six microsatellite loci used in this study were able to distinguish the five high altitude *Rhodohypoxis* species from one another. The markers also clearly distinguished *Rhodohypoxis* species from *H. parvula* var. *parvula*. Using the proposed F_{ST} classes of Hartl et al. (1997) (F_{ST}< 0.05 = little genetic differentiation; 0.05–0.15 = moderate genetic differentiation; 0.15–0.25 = good genetic differentiation; > 0.25 = great genetic differentiation), moderate to great genetic differentiation was recorded between the species in this study. Despite the strong population differentiation among species, patterns of interbreeding and introgression emerged at all three of the study sites sampled.

The highest F_{ST} value for nearly every species (except *H. parvula* var. *parvula*) was between that taxon and *R. deflexa* – indicating that *R. defelxa* is the most genetically differentiated of the species in this study. *Rhodohypoxis defelexa* also formed a cluster far away from the rest of the species in the DAPC (Figure 18). These data are supported by the ecogeographic and phenological barriers reported by Hilliard & Burtt (1978) between this taxon and the rest of the *Rhodohypoxis* species represented in the current study – it is the only species that grows in marshy turf, and that starts flowering from late December to February when most of the other species are at the end of their flowering period. Nevertheless, there were a few instances of detected introgression among *R. defelexa* individuals at Sani Top in the STRUCTURE analysis (Figure 15). One instance involved an *R. rubella* (designated as RT22) individual that appeared to be pure morphologically (here defined as 'pure presenting'), which, according to STRUCTURE, coincided with the *R. rubella* genetic cluster (~90%) and *R. deflexa* genetic cluster (~10%). The other two instances involved 'pure presenting' *R. baurii* var. *confecta* individuals. One individual, designated as CT16, was made up of ~90% *R. baurii var. confecta* and ~10% *R. deflexa*, while the other, designated as CT17, was made up of ~70% *R. baurii* var. *confecta* and ~30% *R. deflexa*. Interestingly, the putative hybrid (HT1) designated as *R. baurii* var. *confecta* × *R. deflexa*, comprises ~95% *R. incompta* alleles, ~4% *R. rubella* alleles, and only ~1% *R. deflexa* alleles.

At near Sani Top, while there was minimal evidence of interbreeding between *R. incompta* and *R. deflexa*, but we found several instances of hybridization between *R. incompta* and *R. baurii* var. *confecta*. Specifically, we identified three individuals as *R. baurii* var. *confecta* (CT10+CT12+CT18) and one as a potential hybrid between *R. baurii* var. *confecta* and *R. rubella*/*R. incompta* (HT5). Additionally, we observed hybridization between *R. incompta* and *R. rubella*, with two individuals showing pure *R. rubella* morphology (RT2 + RT10), one pure *R. incompta* morphology (IT6), and one designated as a putative hybrid

between *R. baurii* var. *confecta* and *R. rubella*/*R. incompta* (HT6). These findings not only support Hilliard & Burtt's (1987) hypothesis that *R. incompta* and *R. rubella* can interbreed at Sani Top, but also confirm our hypothesis that such hybridizations would mostly go unnoticed. This finding also marks the first direct evidence of hybridization between *R. incompta* and *R. baurii* var. *confecta*. Notably, the three putative hybrids between *R. baurii* var. *confecta* and *R. rubella*/*R. incompta* (HT2, HT3, and HT4) showed genetic profiles that were consistent with pure *R. incompta*.

At both the near Sani Top and the Sentinel Peak study sites, instances of admixture were detected between *R. rubella* and *R. baurii* var. *confecta* – representing the first direct evidence for admixture between these two taxa. The individual from near Sani Top, a morphologically pure *R. rubella* (RT19), was made up of ~60% *R. rubella* and ~40% *R. baurii* var. *confecta* while the other individual from Sentinel Peak, a putative *R. baurii* var. *confecta* × *R. rubella* hybrid (RS10; Figure 23), comprised ~53% *R. baurii* var. *confecta* and ~47% *R. rubella* genotypes. The absence of *R. rubella* individuals from the Langalibalele Pass study site in the current study prevented us from assessing whether there was admixture between it and *R. baurii* var. *confecta* at that locality. It also prevented us from substantiating the claim by Hilliard & Burtt (1978) with molecular evidence that the Langalibalele Pass *R. rubella* population is introgressed with *R. thodiana*. Nevertheless, both the STRUCTURE analysis and the DAPC suggested extensive gene flow between *R. thodiana* and *R. baurii* var. *confecta* (the other suspected putative parent of *R. thodiana*) at the Langalibalele Pass study site– suggesting that *R. thodiana* might represent a hybrid swarm between *R. baurii* var. *confecta* and *R. rubella* rather than a hybrid species.

Intergeneric hybridization at Sentinel Peak

It is common to find sympatric populations of *R. baurii* var. *confecta* and *H. parvula* var. *parvula* in the DAC, and it has long been known that these two taxa hybridize in the wild, even though there are clear morphological differences between their flowers (Figure 1, and Hilliard & Burtt, 1978). Sentinel Peak is no exception to this based on the discovery of a putative *R. baurii* var. *confecta* × *H. parvula* var. *parvula* hybrid here (Singh, 2009), and with near complete overlap between their flower phenologies (Figure 20), there are no clear prezygotic barriers between these two taxa that are strong enough to prevent hybridization. Additionally, a previous study based on cpDNA markers indicated that individuals of these two species from Sentinel Peak were genetically similar and could not be genetically

distinguished using plastid markers (Kocyan et al., 2011; Birch & Kocyan, 2021). However, Sentinel Peak *R. baurii* var. *confecta* was found to be diploid (Mtileni et al., 2024) with a DNA index of 1.79–1.88, while Sentinel Peak *H. pavula* var. *parvula* is likely tetraploid (Niemann et al., 2023) with a DNA index of 3.85–4.02 (approx. double the DNA index of Sentinel Peak *R. baurii* var. *confecta*). Even though other studies have reported gene flow between different ploidal levels in plant systems (Wang et al, 2020; Schmickl & Yant, 2021; Cao et al., 2023), this, together with the fact that no putative intermediates could be located at the Sentinel Peak location during the course of the current study, prompted me to search for evidence for gene flow using microsatellite markers as well as undertaking intergeneric crossing experiments between these two taxa.

The population genetic data confirmed the presence of cryptic intergeneric introgression. A single individual, designated as CS2, that appeared to be pure *R. baurii* var. *confecta* showed signs of admixture with *H. parvula* var. *parvula*. According to STRUCTURE, CS2 is made up of ~70% *R. baurii* var. *confecta*, ~28% *H. parvula* var. *parvula* and ~2% *R. rubella* (negligible). Another individual, designated as PS12, originally identified as being pure *H. parvula* var. *parvula*, showed signs of admixture with *R. rubella*. According to STRUCTURE, PS12 is made up of 65% *H. parvula* var. *parvula*, 26% *R. rubella*, and ~9% *R. baurii* var. *confecta*. It is highly unlikely that *H. parvula* var. *parvula* and *R. rubella* would be able to directly hybridize due to strong ecogeographic isolation between them – *H. parvula* var. *parvula* occurs 1900 m.a.s.l to 2200 m.a.s.l (Niemann et al., 2023)., while *R. rubella* usually occurs 2400 m.a.s.l. to 3300 m.a.s.l. (Hilliard & Burtt, 1978). The substantial proportion of *R. baurii* var. *confecta* present (which can be found growing above 2000 m.a.s.l. at Sentinel Peak), however, gives a clue as to what PS12 most likely represents – admixture between an *R. baurii* var. *confecta* × *R. rubella* hybrid and *H. parvula* var. *parvula*. Hilliard & Burtt (1978) documented several instances where putative *Rhodohypoxis* hybrids could be found growing in the intermediate zone between the two putative parent taxa across the DAC. If *R. baurii* var. *confecta* × *R. rubella* hybrids follow a similar pattern, this hybrid taxon would provide a bridge for overcoming the ecogeographic barrier preventing gene flow between *R. rubella* and *H. parvula* var. *parvula* at Sentinel Peak. Further work needs to be done, however, to test this hypothesis. Overall, though, little introgression was detected between *R. baurii* var. *confecta* and *H. parvula* var. *parvula* at the Sentinel Peak study site – all other individuals belonging to either of these two taxa showed admixture levels of less than 10%.

During the course of this study, another population of *H. parvula* var. *parvula* was found growing sympatrically with *R. baurii* var. *confecta* and *R. thodiana* at Langalibalele Pass. Unlike at Sentinel Peak, though, this *H. parvula* var. *parvula* population had the same DNA index as the co-occurring diploid *R. baurii* var. *confecta* (Theron et al., unpublished data). Once again, no putative *R. baurii* var. *confecta* × *H. parvula* var. *parvula* hybrids could be detected at this study site based on phenotype. Nevertheless, the STRUCTURE analysis indicated that admixture was also taking place here to a somewhat greater extent, with four individuals designated as *R. baurii* var. *confecta* (CL6, CL7, CL9 and CL14) displaying admixture with *H. parvula* var. *parvula*. The most admixed individual appeared to be CL14 with 37% admixture with *H. parvula* var. *parvula*, was also made up of 46% *R. thodiana* and only 17% *R. baurii* var. *confecta*. In fact, all these individuals, except CL9, showed some evidence for admixture with *R. thodiana* as well. They likely represent admixture between the *R. baurii* var. *confecta* × *R. thodiana* hybrid individuals and *H. parvula* var. *parvula*. The phenomenon known as triad hybridization occurs when a single species serves as a genetic intermediary, allowing genes (alleles) to transfer between two other species that may not hybridize frequently or at all (Grant & Grant, 2020) and has been reported in plant systems before (Hersch & Roy, 2007; Fogelqvist et al., 2015). Based on these findings, it is possible that *R. thodiana* serves as such a conduit species in this system – allowing gene flow between *R. baurii* var. *confecta* and *H. parvula* var. *parvula*.

Following the extensive evidence for admixture between *R. baurii* var. *confecta* and *R. thodiana* at Langalibalele Pass, and the potential role this could play in facilitating intergeneric hybridization at this study site, it was decided that the crossing experiments between *R. baurii* var. *confecta* and *H. parvula* var. *parvula* should be conducted by only using individuals from the Sentinel Peak study site. However, careful genetic screening of *R. baurii* var. *confecta* individuals from Langalibalele Pass could be done, which would allow for studying of the effect of different DNA ploidies on hybridization success between *R. baurii* var. *confecta* and *H. parvula* var. *parvula* in a future study. Furthermore, studying intergeneric hybridization at the Sentinel Peak study site is of considerable taxonomic significance. *Rhodohypoxis baurii* var. *confecta* and *H. parvula* var. *parvula* individuals from this locality were used in the phylogenetic studies of Kocyan et al. (2011) as well as Birch & Kocyan (2021). The lack of genetic differentiation, as well as the *Hypoxis filiformis* Baker individual included in both studies being sister to the rest of the main clade containing both genera, prompted these workers to propose that *Rhodohypoxis* should be sunk into *Hypoxis*.

Studying the permeability of post-pollination reproductive barriers at this study site might contribute to the continuing discussion regarding the discreteness of *Rhodohypoxis* as a genus.

Hand pollination experiments test species reproductive isolation

The present study found complete self-incompatibility in *H. parvula* var. *parvula*, facultative self-compatibility in *R. baurii* var. *confecta*, and successful crossing between *R. baurii* var. *confecta* and *H. parvula* var. *parvula* from Sentinel Peak with a strong asymmetrical barrier to gene exchange – crosses were only successful when *H. parvula* var. *parvula* was the pollen donor. A meta-analysis done by Tiffin et al. (2001) found that plant species (across numerous lineages) frequently exhibit asymmetry in reproductive isolation and that the self-compatible seed parent usually has a greater seed set than the self-incompatible one – consistent with self-compatible species being more receptive to foreign pollen than self-incompatible ones. Although total hybrid seed formation (three seeds/cross) was lower than the seed formation from *H. parvula* var. *parvula* intraspecific crosses (four seeds/cross) and higher than *R. baurii* var. *confecta* intraspecific crosses (two seeds/cross), hybrid germination rate was substantially lower than those of the positive controls (intraspecific crosses) – consistent with a decrease in F1 viability. Due to it taking ~ three years to cultivate *R. baurii* var. *confecta* and *H. parvula* var. *parvula* from seed to flowering, it was not possible to assess F1 fertility or F2 fertility and viability in this study – an aspect that should be explored in future.

Due to the hybrid crosses being between diploid *R. baurii* var. *confecta* seed parents with a DNA index of 1.79–1.88 and polyploid *H. pavula* var. *parvula* pollen donor with a DNA index of 3.85–4.02 (the DNA indices for all individuals included in the crossing study was checked prior to commencement of the experiment), it was anticipated that the hybrid offspring would have an intermediate DNA index ~2.50–2.80. However, the hybrid offspring had a DNA index 1.73–1.80, which is consistent with the DNA index of the diploid seed parent *R. baurii* var. *confecta*. Negative controls (emasculation of immature buds without fertilization) were put in place from the start of the experiment. This effectively ruled out self-fertilization and apomixis as an explanation for these flow cytometry findings, since bud emasculation without pollination completely prevented seed set in this taxon.

A potential explanation for how these flow cytometry results were possible, came from measuring pollen grains that were originally stained for the pollen viability assays. When measuring the pollen grains for Sentinel Peak *H. parvula* var. *parvula* samples, three

out of the fifteen samples (20%) had pollen that was on average smaller than what was standard for the Sentinel Peak *H. parvula* var. *parvula* population. No apparent decrease in pollen viability was noted for these samples when compared to the other samples from this population. Niemann et al. (2023) showed that the *H. parvula* var. *parvula* population from Monk's Cowl had a DNA index around half that of the Sentinel Peak population (DNA index = ~1.95) – the lowest recorded DNA index to date for this taxon. Accordingly, pollen samples from Monk's Cowl were also measured and compared to those from Sentinel Peak. A significant positive correlation was observed between the mean pollen size values and DNA index of the samples. It was also observed that three samples with smaller pollen grains from the Sentinel Peak population (P3, P8, and P13) overlapped more with the pollen samples from Monk's Cowl plants with smaller DNA indices than with the other twelve samples from polyploid Sentinel Peak plants.

These data suggest that although these three pollen samples from polyploid *H. parvula* var. *parvula* Sentinel Peak plants with a verified DNA index of between 3.93 and 4.11, their grain size corresponds better with lower DNA indices from pollen samples of Monk's Cowl *H. parvula* var. *parvula* plants. This lower DNA index is comparable to the DNA index of diploid *R. baurii* var. *confecta* Sentinel Peak plants and likely explains how this diploid-polyploid cross could have resulted in offspring with a low DNA index. This disparity in pollen size likely also contributes to the low F1 viability, as it is possible that only intergeneric Sentinel Peak crosses where *H. parvula* var. *parvula* individuals like P3, P8, and P13 were involved produce seed that could germinate – rendering the otherwise strong ploidy barrier between these two taxa semi-permeable. Diploid-polyploid crosses that result in diploid-presenting progeny have rarely been recorded by scientists, but when detected and investigated further, is usually linked to double-reduced polyploid pollen (Hanneman & Peloquin, 1968; Kamiri et al., 2009; Aleza et al., 2012; Garavello et al., 2019). The strong asymmetrical barrier to gene flow coupled with the likely requirement of a Sentinel Peak *H. parvula* var. *parvula* polyploid that produces double-reduced pollen for successful crossing is also consistent with high post-pollination reproductive isolation. These findings corroborate the low levels of admixture detected at Sentinel Peak between these two taxa in the STRUCTURE analysis, and the rarity of locating putative *R. baurii* var. *confecta* × *H. parvula* var. *parvula* hybrids based on morphology.

We compared the pollen size measurements of diploid *R. baurii* var. *confecta* with tetraploid *R. baurii* var. *platypetala* from Minerva Private Nature Reserve (PNR) to identify

any similar patterns of pollen size variation. Our analysis revealed a significant positive correlation between mean pollen size and DNA index, which suggests the absence of outlier pollen grains in our sample. However, we observed potential evidence of unreduced gametes, indicated by pollen grains within the tetraploid *R. baurii* var. *platypetala* pollen that overlap in size with the diploid *R. baurii* var. *confecta* samples. These instances are rare, but may contribute to the asymmetrical gene flow observed between these two taxa.

CHAPTER 5: CONCLUSION

Collectively the data from this study (morphological and molecular) suggest inference that the four of the five high-altitude *Rhodohypoxis* taxa delineated in 1978 by Hilliard & Burtt (*R. baurii* var. *confecta*, *R. deflexa*, *R. rubella*, and *R. incompta*) are indeed distinct taxonomic units of unique evolutionary lineages in accordance with the general lineage species concept (GLSC). Evidence, however, seems to suggest that *R. thodiana* might represent a hybrid swarm between *R. baurii* var. *confecta* (with which it extensively hybridizes) and *R. rubella* at Langalibalele Pass. Therefore, caution is applied to its delineation as distinct here, and further work is suggested before a decision is made on its taxonomic status. This study also demonstrated that although morphological intermediacy was the first line of evidence for hybridization between the high-altitude *Rhodohypoxis* species and *H. parvula* var. *parvula*, it significantly underestimated the degree of interspecific as well as intergeneric admixture. It also often resulted in the misidentification of the species included in crosses. The six microsatellite markers used here (which were originally developed for *R. baurii* varieties) demonstrated remarkable interspecific and even intergeneric transferability (with 100% amplification success across the taxa included in this study) and were crucial in allowing us to report the following hybrids for the first time: *R. baurii* var. *confecta* × *R. rubella*, *R. baurii* var. *confecta* × *R. incompta*, *R. rubella* × *R. incompta*. They also substantiated the following putative hybrids identified by Hilliard and Burtt (1978): *R. baurii* var. *confecta* × *R. thodiana* and *R. baurii* var. *confecta* × *R. deflexa*. Rare complex hybrids made up of more than two species were also detected. Finally, this work demonstrates directly that intrageneric hybridization between diploid *R. baurii* var. *confecta* and polyploid *H. parvula* var. *parvula* from Sentinel Peak are indeed possible – which means that cation needs to be taken when making strong taxonomic inferences from phylogenies that include *R. baurii* var. *confecta* and *H. parvula* var. *parvula* representatives from this study site.

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APPENDIX

Table A1. List of specimens used in the analysis of flowering phenology for *Hypoxis parvula* var. *parvula* and *Rhodohypoxis baurii* var. *confecta*. Abbreviations in brackets at the end of voucher information indicates the herbarium of origin (see bottom row for full names). HS = herbarium specimen, LS = living specimen, POSA = Botanical Database of Southern Africa, GBIF = Global Biodiversity Information Facility.

Species	Date	Source	Location	Voucher (HS; POSA) / identifier (GBIF)
H. parvula v. *parvula*	4 December 1982	HS	Underberg distr., Upper Tributaries of Mkomazi River	*Hilliard & Burtt 15864* (NU)
H. parvula v. *parvula*	4 February 1976	HS	Underberg distr., Bushman's Nek, Thamathu Pass	*Hilliard & Burtt 8912* (NU)
H. parvula v. *parvula*	7 November 1951	HS	Cathedral peak forest research station	*Killick 1539* (CPF)
H. parvula v. *parvula*	17 February 1888	HS	Liddesdale	*Wood 3940* (NH)
H. parvula v. *parvula*	4 November 1977	HS	Underberg distr., Garden Castle	*Hilliard & Burtt 10395* (NU)
H. parvula v. *parvula*	1 December 1947	HS	Kamberg "Game Pass"	*Gordon-Gray 66* (NU)
H. parvula v. *parvula*	1 December 1983	HS	Mpendhle distr., Mulangane ridge	*Hilliard & Burtt 16976* (NU)
H. parvula v. *parvula*	20 November 1976	HS	Underberg distr., Cobham Nature Reserve	*Hilliard & Burtt 9288* (NU)
H. parvula v. *parvula*	7 March 1970	HS	Underberg	*Clarke 16* (PRE)
H. parvula v. *parvula*	10 November 1966	HS	Giants Castle Game Reserve	*Trauseld 683* (NU)
H. parvula v. *parvula*	10 January 1990	HS	Mooi River District	*Balkwill & Balkwill 5194* (J)
H. parvula v. *parvula*	4 February 1924	HS	Dundee, Culvers Weenen	*Rogers 28312* (NGB)
H. parvula v. *parvula*	21 November 1973	HS	Underberg distr., Bushman's Nek	*Hilliard & Burtt 7400* (NU)
H. parvula v. *parvula*	23 December 1975	HS	Polela district, Marwaqa	*Rennie 678* (NU)
H. parvula v. *parvula*	1 December 1890	HS	Weenen County, Natal	*Woods 22265* (NBG)

H. parvula v. *parvula*	3 December 1912	HS	Kokstad, Ensekeni (Insikeni)	*Haygarth 12077* (NH)
H. parvula v. *parvula*	6 January 1999	HS	Free State: Sentinel Peak, start of trail	*Singh 465* (NH)
H. parvula v. *parvula*	14 January 1982	HS	Sentinel car park	*Jacob 4865* (NBG)
H. parvula v. *parvula*	10 November 1980	HS	Mount Sheba Nature Reserve	*Kerfoot 8204* (J)
H. parvula v. *parvula*	18 January 1977	HS	Qacha's Nek dist.; Sehlabathebe National Park	*Hoener 1772* (PRE)
R. baurii v. *confecta*	27 November 2019	GBIF	District de Qacha's Nek, Lesotho	*lougarou 82397942*
R. baurii v. *confecta*	26 January 2014	GBIF	Sani Pass, Mkhomazi Wilderness area	*Wahlberg 11002535*
R. baurii v. *confecta*	23 December 2014	GBIF	Royal Natal National Park	*Piry 11012699*
R. baurii v. *confecta*	5 December 2005	POSA	Between Wakkerstroom and Dirkiesdorp	*Craib 0859628* (PRE)
R. baurii v. *confecta*	15 January 2003	POSA	Sani Top Lodge	*Bester* 3987 (PRE)
R. baurii v. *confecta*	6 January 1999	POSA	Qwaqwa National Park; Sentinel Peak	*Singh 466* (NH)
R. baurii v. *confecta*	31 October 1998	POSA	Paardeplaats	*Lotter 476* (PRE)
R. baurii v. *confecta*	11 December 1998	POSA	Qwaqwa National Park; Sentinel Peak	*Singh 458* (PRE)
R. baurii v. *confecta*	22 February 1997	LS	Sani Plateau, N of Sani lodge	*LEG 125* (E)
R. baurii v. *confecta*	14 December 1988	POSA	Golden gate n. park; Oorbietjiekom	*Gertenbach 9118* (PRE)
R. baurii v. *confecta*	10 December 1988	POSA	Golden gate n. park; Oorbietjiekom	*Gertenbach 8852* (PRE)
R. baurii v. *confecta*	4 December 1987	POSA	Qachasnek; just below border police station	*Strever 200* (PRE)
R. baurii v. *confecta*	4 December 1980	POSA	Underberg dist.; Garden Castle Forest Reserve	*Hilliard 13772* (PRE)
R. baurii v. *confecta*	11 December 1980	POSA	Harrismith; Sterkfonteindam	*Blom 82* (PRE)
R. baurii v. *confecta*	17 January 1977	POSA	Harrismith dist.; Rensburgkop plaas	*Jacobsz 546* (PRE)

R. baurii v. *confecta*	6 January 1977	POSA	Underberg dist.; Sani Pass	*Burtt 9615* (PRE)
R. baurii v. *confecta*	7 November 1976	POSA	Wakkerstroom dist.; Rusfontein farm	*Hilliard 9189* (PRE)
R. baurii v. *confecta*	28 December 1975	POSA	Royal Natal National Park	*Hilliard 8685* (PRE)
R. baurii v. *confecta*	30 December 1975	POSA	Harrismith dist.; Platberg	*Hilliard 8693* (PRE)
R. baurii v. *confecta*	10 January 1974	POSA	Harrismith dist.; Platberg	*Jacobsz 56857* (PRE)
R. baurii v. *confecta*	10 January 1974	POSA	Harrismith dist.; Gibsons dam	*Jacobsz 2526* (PRE)
R. baurii v. *confecta*	29 October 1973	POSA	Lions' river dist.; Dargle farm	*Hilliard 6981* (PRE)
R. baurii v. *confecta*	27 November 1973	POSA	Harrismith dist.; Swinburne	*Jacobsz 545* (PRE)
R. baurii v. *confecta*	22 November 1973	POSA	Underberg dist.; Bushman's Nek	*Hilliard 7432* (PRE)
R. baurii v. *confecta*	31 December 1973	POSA	Sani Top	*Hilliard 5414* (PRE)
R. baurii v. *confecta*	31 December 1973	HS	Sani Top	*Hilliard 5414* (K)
R. baurii v. *confecta*	31 December 1973	HS	Sani Top	*Hilliard 5414* (E)
R. baurii v. *confecta*	31 December 1973	HS	Sani Top	*Hilliard 5414* (MO)
R. baurii v. *confecta*	22 October 1961	POSA	Wakkerstroom dist.; Oshoek	*Devenish 705* (PRE)
R. baurii v. *confecta*	1 November 1961	POSA	Barberton dist.; Angle Station	*Codd 38250* (PRE)
R. baurii v. *confecta*	13 November 1957	POSA	Cathedral Peak	*Goodier 372* (PRE)
R. baurii v. *confecta*	22 November 1957	POSA	Mbabane dist.; Nduma	*Compton 27265* (PRE)
R. baurii v. *confecta*	21 October 1956	POSA	Mbabane dist.; Abner's Farm	*Compton 26108* (PRE)
R. baurii v. *confecta*	16 October 1955	POSA	Mbabane dist.; Ukutula	*Compton 25192* (PRE)
R. baurii v. *confecta*	17 December 1955	POSA	Forbes Reef	*Prosser 1958* (PRE)

R. baurii v. *confecta*	23 January 2023	GBIF	Uthukela	*Sashie889 147523278*
R. baurii v. *confecta*	1 December 1930	POSA	Zoutpansberg dist.; Elim	*Obermeyer 29250* (PRE)
R. baurii v. *confecta*	10 November 1929	POSA	Paulpietersburg dist.; Dumbe mtn.	*Galpin 9665* (PRE)
R. baurii v. *confecta*	5 February 1912	POSA	Great Winterhoek mts.	*Thode 1290* (PRE)
R. baurii v. *confecta*	10 January 1920	POSA	Harrismith; Platberg	*Putterill 38280* (PRE)
R. baurii v. *confecta*	1 January 1894	POSA	Witsieshoek; Bestervlei	*Flanagan 1835* (PRE)
R. baurii v. *confecta*	29 January 2023	GBIF	UKhahlamba / Drakensberg Park	*Hallé 147821553*
R. baurii v. *confecta*	28 January 2023	GBIF	Sani Pass, Underberg	*Riegel 184371520*
R. baurii v. *confecta*	28 October 2023	GBIF	Makhelwane east of Sibebe, eSwatini	*Rebelo 189759052*
R. baurii v. *confecta*	28 October 2023	GBIF	Makhelwane	*Braun 189775086*
R. baurii v. *confecta*	26 October 2023	GBIF	Hhohho Region, Eswatini	*Loffler193597351*
R. baurii v. *confecta*	25 November 2023	GBIF	Kaalvoet Vrou Monument	*Falanga 193195281*
R. baurii v. *confecta*	5 December 2023	GBIF	Maluti A Phofung, Qwa-Qwa, FS	*Mantombi 193192968*
R. baurii v. *confecta*	22 February 2022	GBIF	Uthukela, KZN	*Popp 107272720*
R. baurii v. *confecta*	27 September 2022	GBIF	Pigg's Peak, Hhohho, Eswatini	*Loffler 139326514*
R. baurii v. *confecta*	18 October 2022	GBIF	Mvakwelitje, Mbabane, Eswatini	*Loffler 139233377*
R. baurii v. *confecta*	18 November 2022	GBIF	Sani Pass, Mkhomazi Wilderness area	*Swafford 142302998*
R. baurii v. *confecta*	25 November 2022	GBIF	Thabo Mofutsanyane, ZA-FS	*Leroux 142813480*
R. baurii v. *confecta*	31 December 2022	GBIF	Maluti A Phofung, Qwa-Qwa, FS	*Riegel 332327138*
R. baurii v. *confecta*	25 November 2019	GBIF	Mkhomazi Wilderness area, KZN	*lougarou 82397718*

R. baurii v. *confecta*	28 January 2018	GBIF	Sani Pass	*Warren 48312700*
R. baurii v. *confecta*	18 November 2018	GBIF	Hholoshini Trail, Hhohho, SZ	*Loffler 18474036*
R. baurii v. *confecta*	18 December 2018	GBIF	Thabo Mofutsanyane, Free State	*van Zyl 41870900*
R. baurii v. *confecta*	7 December 2015	GBIF	Thabo Mofutsanyane, FS	*Sieben 106060098*
R. baurii v. *confecta*	4 January 1973	HS	Sehlabathebe	*Bayliss 1550436* (WAG)
R. baurii v. *confecta*	1 September 1958	HS	Sani Pass	*Werdermann 10 0003301* (B)
R. baurii v. *confecta*	8 October 1972	HS	High veld trial to Mbabana, SZ	*Keefe 8059* (RSA)
H. parvula v. *parvula*	27 January 2022	GBIF	Free State	*Smit 105665299*
H. parvula v. *parvula*	23 November 2022	GBIF	Maluti A Phofung, Qwa-Qwa, FS	*Smit 142678370*
H. parvula v. *parvula*	22 December 2022	GBIF	Uthukela, KwaZulu-Natal	*Welz 145057918*
H. parvula v. *parvula*	28 December 2021	GBIF	Mkhomazi Wilderness area, KZN	*christian 104021372*
H. parvula v. *parvula*	29 December 2021	GBIF	Giants Castle Game Reserve	*Helme 105253301*
H. parvula v. *parvula*	14 January 2020	GBIF	Devilshoek Trail, Royal Natal National Park	*Falanga 69410114*
H. parvula v. *parvula*	21 December 2020	GBIF	Uthukela, KZN	*Adcock 69909841*

B=Botanischer Garten und Botanisches Museum Berlin-Dahlem, Zentraleinrichtung der Freien Universität Berlin Herbarium
CPF=Donald Killick Herbarium, Pietermaritzburg
E=Royal Botanic Garden Edinburgh herbarium & Living Plant Collections
J=C.E. Moss Herbarium, School of Animal, Plant and Environmental Sciences, University of the Witwatersrand
K=Royal Botanic Gardens Herbarium, Kew, United Kingdom
MO=Missouri Botanical Garden Herbarium
NBG=South African National Biodiversity Institute (SANBI) Compton Herbarium
NH=South African National Biodiversity Institute (SANBI) KwaZulu-Natal Herbarium
NU= Bews Herbarium, University of KwaZulu-Natal, Pietermaritzburg campus
PRE=South African National Biodiversity Institute (SANBI) National Herbarium
RSA=Rancho Santa Ana Botanic Garden Herbarium
WAG=Wageningen University Nationaal Herbarium Nederland, Wageningen University branch

Table A2. The pollen viability data for *R. baurii* var. *confecta* (C1-C15) and *H. parvula* var. *parvula* (P1-P15) from Sentinel Peak car park.

ID	Total percentage viable	Number of pollen grains counted	Viable	Inviable	ID	Total percentage viable	Number of pollen grains counted	Viable	Inviable
C1	83	384	319	65	**P1**	89	313	280	33
C2	63	1124	691	433	**P2**	75	485	367	118
C3	85	338	286	52	**P3**	77	208	161	47
C4	37	1097	405	692	**P4**	91	278	254	24
C5	94	344	325	19	**P5**	79	606	481	125
C6	86	428	369	59	**P6**	83	623	522	101
C7	24	255	205	50	**P7**	94	653	618	35
C8	86	471	405	66	**P8**	91	366	334	32
C9	58	815	477	338	**P9**	86	686	593	93
C10	81	326	263	63	**P10**	95	804	768	36
C11	96	543	522	21	**P11**	85	378	322	56
C12	95	461	438	23	**P12**	51	255	130	125
C13	90	393	355	38	**P13**	68	574	391	183
C14	95	554	525	29	**P14**	95	497	477	20
C15	95	422	402	21	**P15**	81	159	129	30

Table A3. The pollen diameter (at the wides part of the grain) summary statistics for *R. baurii* var. *confecta* (C1-C15) and *H. parvula* var. *parvula* (P1-P15) from Sentinel Peak.

Sample ID	Mean µm	Median µm	Min µm	Max µm	SD
C1	17.45	17	13	22	1.78
C2	18.16	18	13	25	2.09
C3	18.09	18	13	25	2.02
C4	18.38	19	13	28	1.94
C5	18.82	19	14	24	1.90
C6	17.80	18	13	23	1.93
C7	19.20	19	13	24	1.94
C8	18.19	18	13	24	1.80
C9	17.17	17	12	24	1.78
C10	17.83	18	14	23	1.65
C11	16.81	17	12	21	1.67
C12	16.49	16	12	22	1.76
C13	18.19	18	12	23	1.83
C14	17.34	17	12	23	1.95
C15	17.09	17	10	27	2.13
M1	20.68	21	14	27	1.87
M2	22.39	17	22	27	1.80
M3	20.68	21	14	27	1.87
P1	29.01	29	20	36	2.97
P2	32.00	32	22	42	2.87
P3	25.40	26	19	32	2.14
P4	30.47	31	22	41	3.46
P5	29.05	29	23	37	2.43
P6	31.94	32	23	38	2.50
P7	32.53	33	23	40	2.89

P8	27.30	27	18	39	3.40
P9	31.50	32	22	39	2.61
P10	30.26	30	24	36	2.49
P11	32.04	32	19	48	3.45
P12	31.41	31	24	40	2.77
P13	27.08	27	18	34	2.05
P14	30.88	31	22	40	3.39
P15	31.50	32	23	41	3.59
PD1	23.10	23	18	28	1.92
PD2	23.68	24	18	31	1.93
PD3	23.69	24.0	17	31	2.12

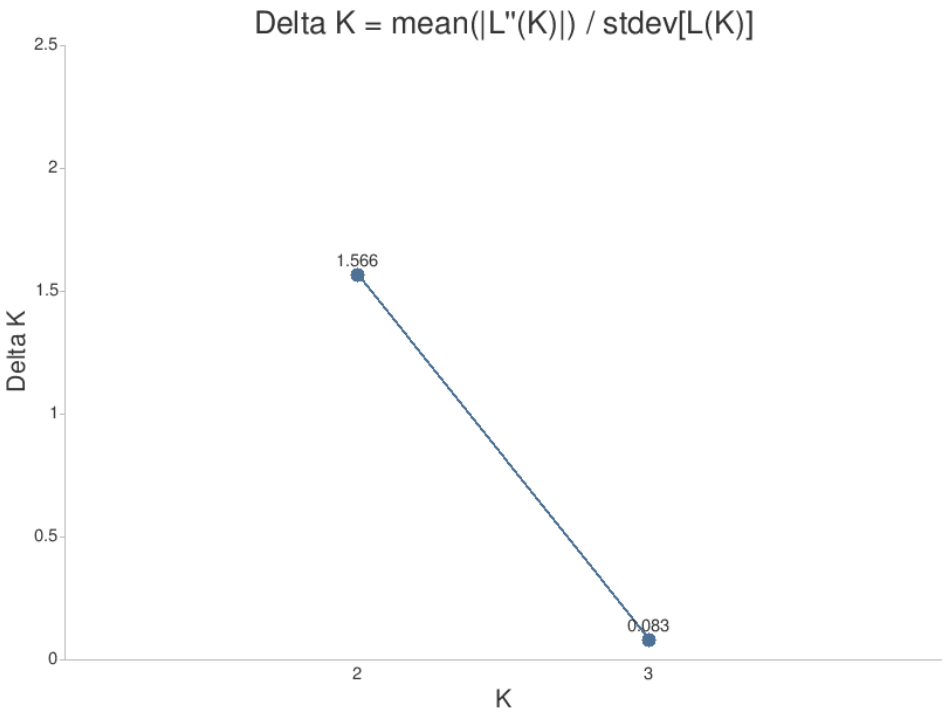

Figure A1. Plot illustrating the Delta K values obtained from the CLUMPAK analysis of the Sentinel Peak microsatellite dataset.

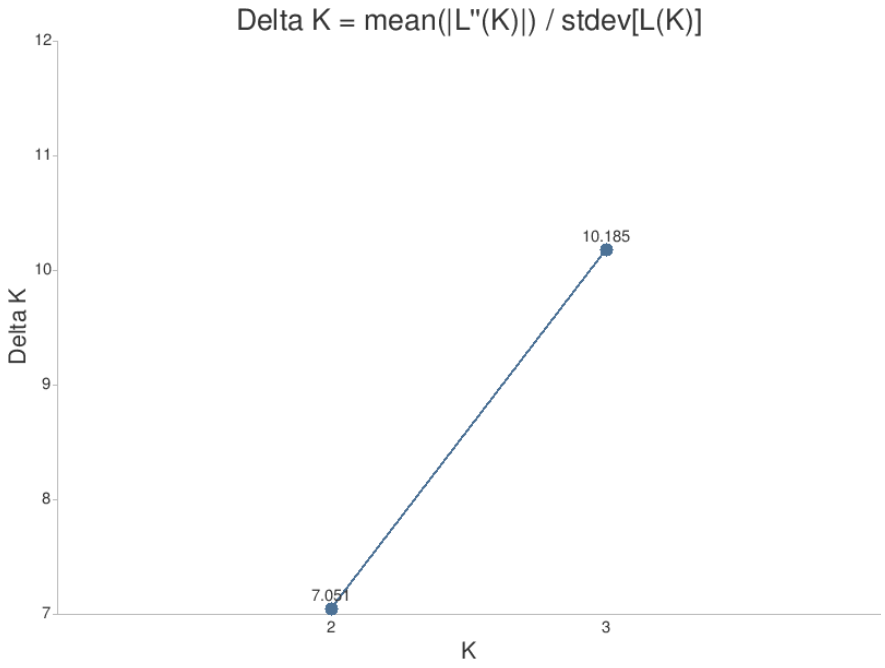

Figure A2. Plot illustrating the Delta K values obtained from the CLUMPAK analysis of the Langalibalele Pass microsatellite dataset.

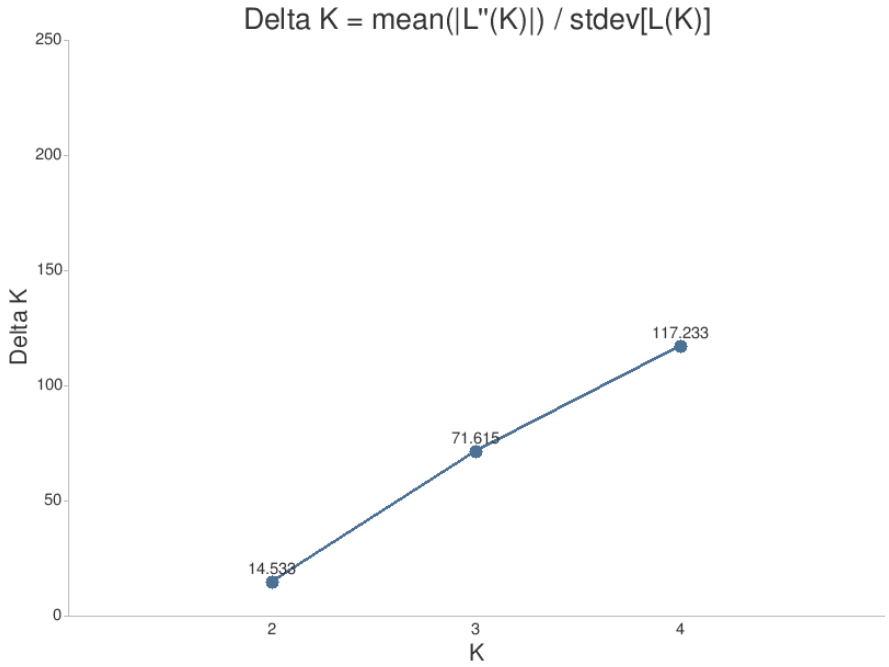

Figure A3. Plot illustrating the Delta K values obtained from the CLUMPAK analysis of the near Sani Top microsatellite dataset.