The curious case of the *Satyrium neglectum* complex: A taxonomic, ecological, and phylogenetic study

By

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PREFACE

The research contained in this dissertation was completed by the candidate while based in the Discipline of Biological Sciences, School of Life Sciences of the College of Agriculture, Engineering and Science, University of KwaZulu-Natal, Pietermaritzburg, South Africa. The research was financially supported by National Research Fund (NRF).

The contents of this work have not been submitted in any form to another university and, except where the work of others is acknowledged in the text, the results reported are due to investigations by the candidate.

Signed: Dr. T. Van der Niet

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DECLARATION: PLAGIARISM

I, Matthew James Rule, declare that:

(i) the research reported in this thesis, except where otherwise indicated or acknowledged, is my original work;

(ii) this thesis has not been submitted in full or in part for any degree or examination to any other university;

(iii) this thesis does not contain other persons' data, pictures, graphs or other information, unless specifically acknowledged as being sourced from other persons;

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a) their words have been re-written but the general information attributed to them has been referenced;

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(vi) this thesis is primarily a collection of material, prepared by myself, published as journal articles or presented as a poster and oral presentations at conferences. In some cases, additional material has been included;

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ABSTRACT

Classical herbarium taxonomy uses a combination of morphological features from pressed specimens and distribution information as data in species delimitation. Whilst morphology is critical to species delimitation, additional information on phylogenetic relationships and field-based studies of ecology can inform taxonomic decisions. *Satyrium neglectum* Schltr. is a highly variable taxon currently comprising two subspecies, ssp. *neglectum* (Schltr.) A.V. Hall and ssp. *woodii* (Schltr.) A.V. Hall both of which are widely distributed in subtropical southern and eastern Africa. Variation in morphology and differences in apparent pollination systems, both between the subspecies, and within ssp. *neglectum* suggests the presence of two taxa within the current concept of the latter. This information, as well the exclusive reliance on herbarium-based taxonomy in the most recent species revision, formed the background to this re-evaluation of the systematics of the complex.

A combination of detailed morphometrics and molecular phylogenetics demonstrated that the current concept of *S. neglectum* ssp. *neglectum* is inaccurate. A principal components analysis using traits measured from herbarium records from across the range, and ethanol-preserved flowers from South Africa, confirmed the presence of two morphologically distinct clusters within ssp. *neglectum*. Representatives from these two forms are separated along an elevation gradient. Bayesian inference using a combination of plastid and nuclear DNA sequence data revealed incongruent topologies. The nuclear topology is not consistent with current taxonomic boundaries, and revealed a separation between the high-elevation form and the low-elevation form of *S. neglectum* ssp. *neglectum* as well as ssp. *woodii*. The plastid data set also confirmed the separation of the high-altitude form of ssp. *neglectum*, but otherwise the topology reflects geography, as southern African accessions are separate from eastern African accessions.

Floral scent was investigated in combination with published and novel pollinator observations in the *Satyrium neglectum* species complex. Floral scent composition and emission rates are mostly representative of associated pollination syndromes in previously published pollination work, with the exception of the high-elevation form. Published observations of ssp. *woodii* indicate that the species is pollinated by amethyst and the greater-double-coloured sunbirds, and the scent composition and emission rate is largely reflective of this. In addition, published observations showed a system of butterfly pollination in the low-elevation form of ssp. *neglectum* and long-proboscid fly pollination in the high-elevation form. However, new night-

time observations revealed additional pollination by settling moths in the high elevation form. These observations make sense in the context of the scent profile, which is dominated by compounds typically associated with moth pollination, such as phenylethyl alcohol and eugenol. In addition, two settling moths showed electroantennographic responses to the two dominant compounds in the scent bouquet of the high-elevation form. The compounds that dominate the scent of the butterfly-pollinated low elevation form are hexan-1-ol, octan-1-ol and benzyl alcohol, while previously published work on ssp. *woodii* showed that it is virtually unscented, and has emission rates that are markedly lower than other taxa pollinated by insects.

Based on the morphological, phylogenetic and scent chemistry analyses, a case was made for the high-altitude form to be considered a separate taxonomic entity. This is formally described under the new name *Satyrium basutorum*. The analyses suggest that ssp. *neglectum* and ssp. *woodii* should be retained as subspecies, but a further investigation is required to identify the status of the eastern African synonyms *Satyrium sceptrum* and *S. neglectum* var. *brevicalcar*.

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CHAPTER 1 : INTRODUCTION AND LITERATURE REVIEW

1.1 Introduction

Humanity has an innate need to categorise various aspects of the natural world to allow for the creation and organisation of ideas that are more palatable. The scientific method of taxonomy, which is defined as the naming and classifying of organisms into groups based on shared characteristics, illustrates this, and is a vital branch in biology. The correct naming of species is of fundamental importance for the complete understanding of biodiversity, the creation of tailored conservation methods, and is necessary to communicate throughout the scientific community at large (Raven, 2004; Henderson, 2005; Bebber et al., 2010; Sosef et al., 2020).

Before the development of scientific testing, plants and animals were named and categorised according to the similarity in their morphological structure (Simpson, 1951; Mishler and Budd, 1990; Raven, 2004; Henderson, 2005; Sosef et al., 2020). The classic binomial nomenclature system still in use today, involves a double naming system, in which a species is given what Carl Linnaeus called a "trivial" name, which is preceded by the genus (Linnaeus and Willdenow, 1799). The Linnean binomial classification system provides a useful and rigid system for naming organisms, and the categorisation and delimitation of species, however, as new technology has evolved, so too has our understanding of what a species is and how it should be delineated.

The oldest methodology used in species delimitation revolved around the examination of morphological similarity between specimens (Morphological Species Concept). Closely associated to the former, is the typological species concept which emphasises the need to have a type specimen which represents the characteristic and diagnostic features of species which are used as a reference in the identification of further specimens (Mayr, 1976, 1996). Both of these concepts rely heavily on morphological similarity and dissimilarity to distinguish species, but neither of these concepts takes the evolutionary relationships nor the process of ecological adaptation, analogous traits or reproductive isolation into consideration (Simpson, 1951; Mishler and Budd, 1990). Many of the inherent problems in morphology-based taxonomy were somewhat resolved with the implementation of phylogenetic analysis (Freudenstein and Rasmussen, 1999; Chase, 2005), as phylogenetic analysis using molecular data provided an avenue to identify morphological homoplasious traits which had arisen through convergent

evolution, which infer false phylogenetic relationships (Endress, 1996; Sanderson and Hufford, 1996; Freudenstein and Rasmussen, 1999; Kress et al., 2002; Chase, 2005; Wu et al., 2015).

1.2 Herbarium-based Taxonomy

Additional problems of morphology-based taxonomy can be identified in taxonomic studies which exclusively use herbarium records as a source for delimitation. Herbaria reflect the shared knowledge and collection of hundreds of years of both scientists and botanical enthusiasts. The historical and scientific importance of a herbarium should not be underestimated both in its representation of the historical and geographic range of a species' distribution and providing a font of new as yet-undescribed species (Bebber et al., 2010; Botes et al., 2020; Sosef et al., 2020). However, the exclusive use of herbarium records in taxonomy is limited due to a variety of problems.

The first problem relates to a large number of herbarium records being incomplete and this is especially true in older specimens, in addition to the fact that distinguishing characters in certain taxa are impossible to collect, or are sometimes overlooked if their importance in delimitation is unknown (Gentry, 1989; Bebber et al., 2010). An example of this can be found in the case of two *Maquira* species, where herbarium records did not reflect differences in latex colour or canopy structure, which are both key characteristics in delimitation and easily allow for distinguishing between the two species in the field (Gentry, 1989). Inaccurate grouping of taxa based on herbarium records is further exacerbated in the loss or distortion of critical traits in the mechanical pressing and storage of collected material (Parnell et al., 2013), leading to an underestimation of biodiversity in collected specimens (Bebber et al., 2010; Botes et al., 2020).

Many taxonomic traits which are used in species delimitation are also related to characteristics measured in the flower, including dimensions of the perianth, and traits related to the reproductive organs in flowers (Grant, 1949; Anderson et al., 2002). Diversity in floral traits is largely characterised by changes in available pollinator types (Stebbins, 1970; Johnson and Steiner, 1997; Van der Niet et al., 2014), with limited pollinator distributions being largely responsible for shifts in floral traits through pollinator-mediated trait selection, eventually culminating in reproductive isolation and speciation (Johnson, 1997b). Despite the link between floral traits and pollinator-mediated selection, many important traits related to

pollination, such as floral scent composition, which can be quantified in the field, are not included in taxonomic studies. The link between floral scent chemistry and phylogenetics has been well documented, and has been used to infer phylogenetic relationships (Dahl et al., 1990; Azuma et al., 1999; Jurgens et al., 2003; Steiner et al., 2011). Although information recorded by the collector can provide some means of examining these traits in herbarium records, it can only be vaguely described using abstract words such as "sweet" or "pleasantly scented". In species complexes, where morphology may not clearly distinguish between forms, an integrative approach should be taken, with the inclusion of traits associated with pollination and ecology to provide clarifications in the re-examination of taxonomic groups, in addition to rigorous phylogenetic analyses (Dayrat, 2005; Padial et al., 2009; Pace et al., 2019; Botes et al., 2020).

1.3 Pollination syndromes and pollinator-mediated trait selection

The enormous floral diversity in angiosperms can be explained by the interaction between pollinators and flowers. As was so aptly described in Galen (1999, p. 631): "Pollinators have the motive (energy and nutrition) and the means (pollen transfer, a key step in plant sexual reproduction) to exert selection on the floral features of their host plants". The dependence on pollinators for the transferal of pollen consequently allows for pollinator-mediated trait selection to dictate both floral morphology, colour and scent composition (Waser and Campbell, 2004; Mant et al., 2005; Johnson, 2006; Schiestl and Schluter, 2009). Selection on floral traits can either favour generalization, in which floral morphology or signalling cues neither excludes nor attracts specific pollinator groups, or selection can favour the specialisation of floral traits such that only interactions with a specific pollinator or pollinator type can pollination be efficient, and genetic exchange can occur (Faegri and van der Pijl, 1979; Waser and Price, 1993; Fenster et al., 2004).

Analogous features of floral structure, colour and scent composition are representative of convergent evolution in flowers to utilise and exploit the sensory ability, behaviour and morphology of available pollinator groups for pollen transferral (Faegri and van der Pijl, 1979; Fenster et al., 2004). Pollination syndromes are thus described as a suite of characteristics which categorises floral traits and their association with a group or a type of pollinator (Faegri and van der Pijl, 1979; Fenster et al., 2004). Floral traits can become highly specialised and unique to different systems leading to the formation of a co-dependence between pollinators

and flowers (Johnson and Steiner, 2000; Fenster et al., 2004), often resulting in highly specialised interactions which can limit pollen transfer and genetic exchange to specific interactions, thus excluding numerous pollinator groups (Galen, 1999; Johnson and Steiner, 2000; Fenster et al., 2004).

Floral scent in flowers has been recorded as a functional trait in numerous pollination systems, with the exception of birds and long-proboscid flies (Proctor et al., 1996; Goldblatt and Manning, 2000; Dobson, 2006; Farré-Armengol et al., 2015). Floral scent compounds are diverse, and signal not only the presence of nutritional reward (Son et al., 1996; Raguso, 2004), but can also become highly unique in extremely specialised pollination syndromes (Dobson, 2006; Johnson and Jurgens, 2010; Van der Niet et al., 2011). The scent composition and emission rates of flowers have been known to be extremely important in various plant-pollinator interactions, and may provide key differences in closely related taxa (Knudsen and Tollsten, 1993; Dobson, 2006; Ayasse, 2007; Shuttleworth and Johnson, 2009; Johnson and Jurgens, 2010).

1.4 The genus *Satyrium*

Satyrium Sw. (subtribe *Satyriinae) is* widely distributed and variable terrestrial orchid genus which is comprises approximately 93 species (Hall, 1982; Van der Niet et al., 2005; Van der Niet and Cribb, 2006), and has a wide distribution range, predominantly in the interior of Africa, along the Rift Valley, into the eastern regions of the continent through Malawi and Tanzania, and with some species occurring on Madagascar and in southeast Asia. The genus also has a centre of diversity in southern Africa, with a range that extends from the winter-rainfall areas in the Greater Cape Floristic Region (Born et al., 2007), along the Drakensberg Mountain range in the Eastern Cape, into KwaZulu-Natal and Lesotho, with a further extension into Mpumalanga and further radiations in the grasslands of South-Central, and Eastern Africa (Hall, 1982; Johnson et al., 2011) The genus is especially species rich in the Cape Floristic Region, which is a major hotspot for diversity, not only for Orchidaceae, but numerous other plant families (Goldblatt and Manning, 2002; Verboom et al., 2009).

Satyrium species are characterised by their zygomorphic twin-spurred, non-resupinate flowers (Kurzweil, 1996; Kurzweil and Linder, 1999). Floral trait variation in *Satyrium* has been well documented, and often includes distinction in the length of the spur, and rostellum, as well as

differences in colour, scent composition and emission rates (Johnson, 1996; Johnson et al., 2007; Johnson et al., 2011; Van der Niet et al., 2011; Van der Niet et al., 2015b). Differences in floral traits within *Satyrium* are associated with differences in pollination systems, resulting from pollinator-mediated trait selection and adaptive radiation (Johnson, 1997b).

Numerous pollination systems have been recorded in *Satyrium*, including but not limited to: beetles and wasps (Johnson et al., 2007), flies, butterflies, moths (Johnson, 1997a, 1997b; Johnson et al., 2011) and birds (Johnson, 1996; Johnson et al., 2011; Johnson and Van der Niet, 2019). For each of the aforementioned pollinator groups, there is a close association between a set of floral traits, related to pollinator morphology, behaviour or sensory capability (Faegri and van der Pijl, 1979; Armbruster et al., 2000). As such, floral scent in *Satyrium* is highly variable, specialised and closely associated with pollination systems. Specialised scent systems in the genus include: *Satyrium pumilum,* which emits similar scent compounds to those found in carrion, and whose visitors closely match those found on animal carcasses, indicating selection for pollination by carrion flies (Van der Niet et al., 2011). In addition, a combination of floral traits in *Satyrium microrrhynchum* allows pollination by both wasps and beetles and shows adaptation to scent and floral morphology to accommodate both pollinator types (Johnson et al., 2007). In addition, a lack of scent has also been noted in at least two *Satyrium* species which were found to be pollinated by birds, including *Satyrium rhodanthum* (Van der Niet et al., 2015a) and *Satyrium neglectum* ssp. *woodii* (Johnson, 1996; Johnson and Van der Niet, 2019).

1.5 *The Satyrium neglectum* complex

Satyrium neglectum Schltr. is considered a highly variable species complex that is comprises two subspecies: *S. neglectum* ssp. *neglectum* and *S. neglectum* ssp. *woodii* (Hall, 1982). The species is known to occur throughout southern and eastern Africa, with a distribution which occurs from the southern peaks of the Drakensberg, up into in Tanzania, Malawi and Kenya (Hall, 1982). Intra-specific morphological variation in ssp. *neglectum*, led to the identification of two forms, separated by distribution in differing elevations, with these two forms differing in spur length, among other floral traits (Figure 1.1) (Johnson et al., 2011). While the two forms differ in apparent elevation, some populations, such as those at Sani Pass are relatively close geographically, with the low-elevation form occurring at the base of the pass and the high-elevation form occurring at the summit. Populations of the southern African high-elevation

form in the highlands of Lesotho and Kwa-Zulu Natal were explicitly linked to the short-spurred Tanzanian synonym, var. *brevicalcar* Summerh., though they were found to only be negligibly different from long-spurred var. *neglectum*, and both were relegated to synonymy as ssp. *neglectum* (Summerhayes, 1966; Hall, 1982). In addition, *Satyrium sceptrum*, which was relegated to synonymy in ssp. *woodii* (Hall, 1982), is still sometimes referred to as a separate taxonomical entity, that is considered to be morphologically identifiable distinguishable in the field, when compared with ssp. *woodii* (Pope, 1995; Van der Niet and Linder, 2008; Johnson et al., 2011).

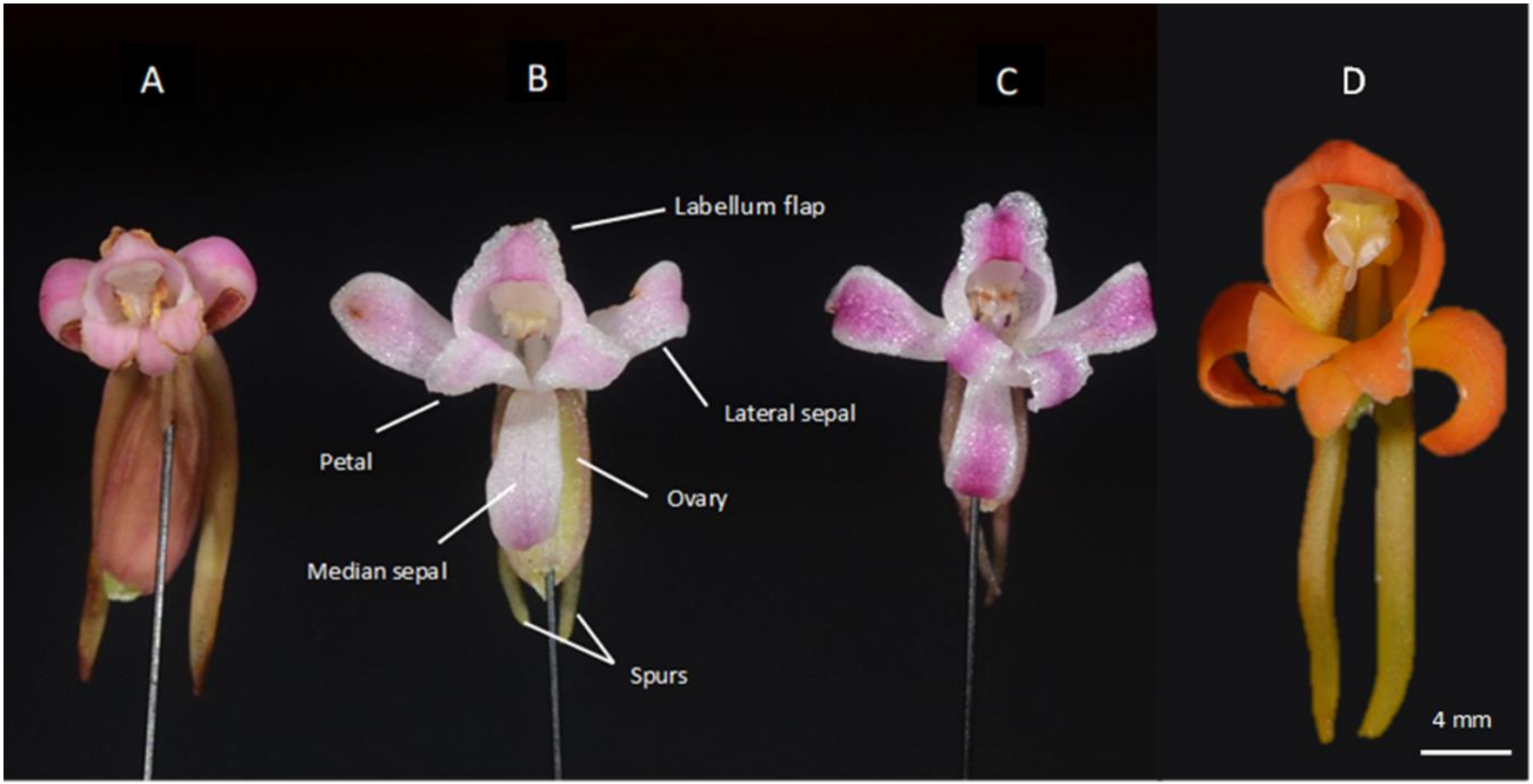

Figure 1.1: Flower morphology of the two elevation forms in *Satyrium neglectum* ssp. *neglectum* and ssp. *woodii*. Low-elevation form (A); Gradient of floral colour intensity in the high-elevation form (B and C) and *S. neglectum* ssp. *woodii* (D).

Several studies have investigated the pollination systems in the *S. neglectum* species complex. Observations of bird pollination have been recorded in *Satyrium neglectum* ssp. *woodii* and in the Tanzanian *S. sceptrum* (Van der Niet et al., 2005; Johnson et al., 2011; Johnson and Van der Niet, 2019). The morphology of ssp. *woodii* conforms to traits associated with bird pollination, specifically the orange-red flowers, with long spurs, and little to no scent (Faegri and van der Pijl, 1979; Dobson, 2006). This was corroborated by observations of sunbirds pollinating specimens of *S. sceptrum* in a population in Malawi (Johnson et al., 2011), and a southern African population of *S. neglectum* ssp. *woodii* (Johnson and Van der Niet, 2019).

Published pollinator observations suggest differences between the two elevation forms of *S. neglectum* ssp. *neglectum* in South Africa (Johnson et al., 2011). Observations of the high-elevation form revealed pollination by long-proboscid flies, when individuals of *Prosoeca ganglbaueri* were observed visiting flowers at Mount. Aux-Sources (Johnson et al., 2011). Pollinator observations revealed that the low-elevation form is pollinated by butterflies belonging to either *Pieridae* or *Nymphalidae* (Johnson et al., 2011).The high-elevation form has a distinctly sweet scent to the human-nose, which has so far been found to be insignificant in long-proboscid fly pollination (Goldblatt and Manning, 2000). The presence of a distinct scent, and other traits consistent with the moth pollination syndrome (white, pale flowers), suggest that the published observations in the high-elevation form may be representative of a (1) bimodal pollination system, (2) differences in available pollinators, or (3) characterised by visitation by opportunistic visitors.

In addition to differences among pollination systems, phylogenetic analysis of the *Satyrium neglectum* complex illustrates inconsistencies with the current taxonomic concept of the species. A previous phylogenetic analysis which included accessions of both the high- and low elevation forms of *Satyrium neglectum* ssp. *neglectum* found that despite incongruencies between plastid and nuclear datasets, samples representing the two forms were not monophyletic, as the current taxonomic classification would suggest (Figure 1 of Van der Niet and Linder, 2008). Accessions from the low-elevation form however were sampled from Malawi and Tanzania whereas those of the high-elevation form were sampled from Drakensberg Mountain Range in South Africa. Given the large geographic disjunction between accessions representing the low- and high-elevation forms, these two regions may contain populations whose genetic differentiation is substantial, which may explain the non-

monophyletic grouping between these accessions. Thus, it is necessary to include representatives from populations of the southern African forms to determine if this pattern is consistent across this large geographical range.

The overarching aim of this thesis is to investigate the systematics, pollination ecology and scent chemistry of the *Satyrium neglectum* species complex, given the inconsistencies in the phylogenetic relationships, the exclusive use of herbarium taxonomy in the previous revision of the southern African genera (Hall, 1982), and mismatch in important floral traits and observed pollination systems. This will be undertaken through the examination and subsequent explanation of the variation found in the *Satyrium neglectum* species complex. This thesis will emphasise the importance of the use of a combination of both herbarium and field based-taxonomy. The combination of both traditional methods of morphological taxonomical studies, as well as phylogenetic and comparative scent analysis studies, may demonstrate how the exclusive use of herbarium based-taxonomy and the exclusion of vital phylogenetic and ecological studies may obscure accurate taxonomical estimations.

1.6 Outline of dissertation structure

In Chapter 2, I provide a comparative morphological analysis in the species complex of *S. neglectum,* using both herbarium records from across the species range, and preserved flowers collected from southern African populations. This study revisits the phylogeny of the *S. neglectum* species complex, using both plastid and nuclear datasets and introduces an expanded sampling throughout the distribution of the species complex. This Chapter aims to provide evidence for the delimitation of the components of the *S. neglectum* species complex through both comparative morphological and phylogenetic analysis. The investigation of both is crucial to pinpoint and explain the variation found in this complex.

Chapter 3 is devoted to an investigation into floral scent chemistry of the entire *S. neglectum* species complex, including the high- and low-elevation forms of *S. neglectum* ssp. *neglectum,* as well as *S. neglectum* ssp. *woodii*. This study will focus on the scent characterisation of the taxa, as part of a comparative analysis using both novel and published pollination observations.

Chapter 4 is devoted to the description of *Satyrium basutorum,* which represents the previous high-elevation form in *Satyrium neglectum* ssp. *neglectum,* and provides a species treatment, distribution, and habitat description.

The final chapter makes concluding remarks regarding classical herbarium taxonomy, the description of *S. basutorum* and further demonstrates how an integrative investigation, comprised of a multidisciplinary study is key in species delimitation. This chapter also provides recommendations on additional studies which are necessary to further understand the *S. neglectum* species complex.

1.7 References

Anderson, G. J., Johnson, S. D., Neal, P. R., Bernardello, G., 2002. Reproductive biology and plant systematics: the growth of a symbiotic association. Taxon 51, 637-653.

Armbruster, W. S., Fenster, C. B., Dudash, M. R., 2000. Pollination "principles" revisited: specialization, pollination syndromes, and the evolution of flowers. Det Norske Videnskaps-Akademi. I. Matematisk Naturvidenskapelige Klasse, Skrifter, Ny Serie 39, 179-200.

Ayasse, M., 2007. Chemical mimicry in sexually deceptive orchids of the genus *Ophrys*. Phyton-Annales Rei Botanicae 46, 221-223.

Azuma, H., Thien, L. B., Kawano, S., 1999. Molecular phylogeny of *Magnolia* (Magnoliaceae) inferred from cpDNA sequences and evolutionary divergence of the floral scents. Journal of Plant Research 112, 291-306.

Bebber, D. P., Carine, M. A., Wood, J. R., Wortley, A. H., Harris, D. J., Prance, G. T., Davidse, G., Paige, J., Pennington, T. D., Robson, N. K., 2010. Herbaria are a major frontier for species discovery. Proceedings of the National Academy of Sciences 107, 22169-22171.

Born, J., Linder, H., Desmet, P., 2007. The Greater Cape floristic region. Journal of Biogeography 34, 147-162.

Botes, C., van der Niet, T., Cowling, R. M., Johnson, S. D., 2020. Is biodiversity underestimated by classical herbarium-based taxonomy? A multi-disciplinary case study in *Satyrium* (Orchidaceae). Botanical Journal of the Linnean Society 194, 342-357.

Chase, M. W., 2005. Classification of Orchidaceae in the age of DNA data. Curtis's Botanical Magazine 22, 2-7.

Dahl, Å. E., Wassgren, A.-B., Bergström, G., 1990. Floral scents in *Hypecoum* sect. *Hypecoum* (Papaveraceae): chemical composition and relevance to taxonomy and mating system. Biochemical Systematics and Ecology 18, 157-168.

Dayrat, B., 2005. Towards integrative taxonomy. Biological Journal of the Linnean Society 85, 407-417.

Dobson, H. 2006. Relationship between floral fragrance composition and type of pollinator. Pages 147-198.In D. N , E, P., eds. Biology of Floral Scent. CRC Press, Boca Raton: .

Endress, P. K., 1996. Homoplasy in angiosperm flowers. Homoplasy: the recurrence of similarity in evolution. Academic Press, San Diego, 303-325.

Faegri, K., van der Pijl, L. 1979. The Principles of Pollination Ecology. Pergamon, Oxford.

Farré-Armengol, G., Filella, I., Llusià, J., Peñuelas, J., 2015. Pollination mode determines floral scent. Biochemical Systematics and Ecology 61, 44-53.

Fenster, C. B., Armbruster, W. S., Wilson, P., Dudash, M. R., Thomson, J. D., 2004. Pollination syndromes and floral specialization. Annual Review of Ecology Evolution and Systematics 35, 375-403.

Freudenstein, J. V., Rasmussen, F. N., 1999. What does morphology tell us about orchid relationships? - A cladistic analysis. American Journal of Botany 86, 225-248.

Galen, C., 1999. Why do flowers vary? The functional ecology of variation in flower size and form within natural plant populations. Bioscience 49, 631-640.

Gentry, A., 1989. Herbarium taxonomy versus field knowledge. Is there an attainable solution? Flora Malesiana Bulletin 10, 31-35.

Goldblatt, P., Manning, J. C., 2000. The long-proboscid fly pollination system in southern Africa. Annals of the Missouri Botanical Garden 87, 146-170.

Goldblatt, P., Manning, J. C., 2002. Plant diversity of the Cape region of southern Africa. Annals of the Missouri Botanical Garden, 281-302.

Grant, V., 1949. Pollination systems as isolating mechanisms in angiosperms. Evolution 3, 82-97.

Hall, A. V. 1982. A Revision of the Southern African Species of *Satyrium*. Bolus Herbarium, Rondebosch, Republic of South Africa.

Henderson, A., 2005. The methods of herbarium taxonomy. Systematic Botany 30, 456-459.

Johnson, S., 1997a. Insect pollination and floral mechanisms in South African species of *Satyrium* (Orchidaceae). Plant Systematics and Evolution 204, 195-206.

Johnson, S., 1997b. Pollination ecotypes of *Satyrium hallackii* (Orchidaceae) in South Africa. Botanical Journal of the Linnean Society 123, 225-235.

Johnson, S. D., 1996. Bird pollination in South African species of *Satyrium* (Orchidaceae). Plant Systematics and Evolution 203, 91-98.

Johnson, S. D. 2006. Pollinator-driven speciation in plants. Pages 295-310.In L. D. Harder , Barrett, S. C. H., eds. The ecology and evolution of flowers. Oxford University Press, Oxford.

Johnson, S. D., Ellis, A., Dotterl, S., 2007. Specialization for pollination by beetles and wasps: The role of lollipop hairs and fragrance in *Satyrium microrrhynchum* (Orchidaceae). American Journal of Botany 94, 47-55.

Johnson, S. D., Jurgens, A., 2010. Convergent evolution of carrion and faecal scent mimicry in fly-pollinated angiosperm flowers and a stinkhorn fungus. South African Journal of Botany 76, 796-807.

Johnson, S. D., Peter, C. I., Ellis, A. G., Boberg, E., Botes, C., Van der Niet, T., 2011. Diverse pollination systems of the twin-spurred orchid genus *Satyrium* in African grasslands. Plant Systematics and Evolution 292, 95-103.

Johnson, S. D., Steiner, K. E., 1997. Long-tongued fly pollination and evolution of floral spur length in the *Disa draconis* complex (Orchidaceae). Evolution 51, 45-53.

Johnson, S. D., Steiner, K. E., 2000. Generalization versus specialization in plant pollination systems. Trends in Ecology & Evolution 15, 190--193.

Johnson, S. D., Van der Niet, T., 2019. Bird pollination in an African *Satyrium* (Orchidaceae) confirmed by camera traps and selective exclusion experiments. Plant Systematics and Evolution 305, 477-484.

Jurgens, A., Witt, T., Gottsberger, G., 2003. Flower scent composition in *Dianthus* and *Saponaria species* (Caryophyllaceae) and its relevance for pollination biology and taxonomy. Biochemical Systematics and Ecology 31, 345-357.

Knudsen, J. T., Tollsten, L., 1993. Trends in floral scent chemistry in pollination syndromes: floral scent composition in moth-pollinated taxa. Botanical Journal of the Linnean Society 113, 263-284.

Kress, W. J., Prince, L. M., Williams, K. J., 2002. The phylogeny and a new classification of the gingers (Zingiberaceae): evidence from molecular data. American Journal of Botany 89, 1682-1696.

Kurzweil, H., 1996. Floral morphology and ontogeny in subtribe *Satyriinae* (Fam Orchidaceae). Flora 191, 9-28.

Kurzweil, H., Linder, H. P., 1999. A phylogenetic analysis of the genus *Satyrium* (Orchidaceae). Beitraege zur Biologie der Pflanzen 71, 101-181.

Linnaeus, C., Willdenow, K. L. 1799. Species plantarum : exhibentes plantas rite cognitas ad genera relatas, cum differentiis specificis, nominibus trivialibus, synonymis selectis, locis natalibus, secundum systema sexuale digestas. Impensis GC Nauk, Berolini.

Mant, J., Peakall, R., Schiestl, F. P., 2005. Does selection on floral odor promote differentiation among populations and species of the sexually deceptive orchid genus *Ophrys*? Evolution 59, 1449-1463.

Mayr, E. 1976. Species concepts and definitions. Pages 353-371 Topics in the Philosophy of Biology. Springer.

Mayr, E., 1996. What is a species, and what is not? Philosophy of Science 63, 262-277.

Mishler, B. D., Budd, A. F., 1990. Species and evolution in clonal organisms-introduction. Systematic Botany 15, 79-85.

Pace, M. C., Giraldo, G., Frericks, J., Lehnebach, C. A., Cameron, K. M., 2019. Illuminating the systematics of the *Spiranthes sinensis* species complex (Orchidaceae): ecological speciation with little morphological differentiation. Botanical Journal of the Linnean Society 189, 36-62.

Padial, J. M., Castroviejo-Fisher, S., Koehler, J., Vila, C., Chaparro, J. C., De la Riva, I., 2009. Deciphering the products of evolution at the species level: the need for an integrative taxonomy. Zoologica Scripta 38, 431-447.

Parnell, J., Rich, T., McVeigh, A., Lim, A., Quigley, S., Morris, D., Wong, Z., 2013. The effect of preservation methods on plant morphology. Taxon 62, 1259-1265.

Pope, G. V. 1995. Flora Zambesiaca: Mozambique, Malawi, Zambia, Zimbabwe, Botswana. Volume 11: Part 1. Flora Zambesiaca Management Committee.

Proctor, M., Yeo, P., Lack, A. 1996. The Natural History of Pollination. Collins, London.

Raguso, R. A., 2004. Why are some floral nectars scented? Ecology 85, 1486-1494.

Raven, P. H., 2004. Taxonomy: where are we now? Philosophical Transactions of the Royal Society of London. Series B: Biological Sciences 359, 729-730.

Sanderson, M. J., Hufford, L. 1996. Homoplasy: the recurrence of similarity in evolution. Elsevier.

Schiestl, F. P., Schluter, P. M., 2009. Floral Isolation, Specialized Pollination, and Pollinator Behavior in Orchids. Annual Review of Entomology 54, 425-446.

Shuttleworth, A., Johnson, S. D., 2009. The importance of scent and nectar filters in a specialized wasp-pollination system. Functional Ecology 23, 931-940.

Simpson, G. G., 1951. The species concept. Evolution 5, 285-298.

Son, H. E. D., Groth, I., Bergström, G., 1996. Pollen advertisement: Chemical contrasts between whole-flower and pollen odors. American Journal of Botany 83, 877-885.

Sosef, M. S. M., Degreef, J., Engledow, H., Meerts, P. 2020. Botanical classification and nomenclature - an introduction. Meise Botanic Garden, Belgium.

Stebbins, G. L., 1970. Adaptive radiation of reproductive characteristics in angiosperms, I: pollination mechanisms. Annual Review of Ecology and Systematics 1, 307-326.

Steiner, K. E., Kaiser, R., Dötterl, S., 2011. Strong phylogenetic effects on floral scent variation of oil-secreting orchids in South Africa. American Journal of Botany 98, 1663-1679.

Summerhayes, V., 1966. African orchids: XXX. Kew Bulletin 20, 165-199.

Van der Niet, T., Cozien, R. J., Johnson, S. D., 2015a. Experimental evidence for specialized bird pollination in the endangered South African orchid *Satyrium rhodanthum* and analysis of associated floral traits. Botanical Journal of the Linnean Society 177, 141-150.

Van der Niet, T., Cribb, P., 2006. Additions to the orchid flora of Angola, Tanzania and Zimbabwe. Kew Bulletin, 261-264.

Van der Niet, T., Hansen, D. M., Johnson, S. D., 2011. Carrion mimicry in a South African orchid: Flowers attract a narrow subset of the fly assemblage on animal carcasses. Annals of Botany 77, 981-992.

Van der Niet, T., Jürgens, A., Johnson, S., 2015b. Is the timing of scent emission correlated with insect visitor activity and pollination in long-spurred *Satyrium* species? Plant Biology 17, 226-237.

Van der Niet, T., Linder, H., 2008. Dealing with incongruence in the quest for the species tree: a case study from the orchid genus *Satyrium*. Molecular Phylogenetics and Evolution 47, 154-174.

Van der Niet, T., Linder, H. P., Bytebier, B., Bellstedt, D. U., 2005. Molecular markers reject monophyly of the subgenera of *Satyrium* (Orchidaceae). Systematic Botany 30, 263-274.

Van der Niet, T., Pirie, M. D., Shuttleworth, A., Johnson, S. D., Midgley, J. J., 2014. Do pollinator distributions underlie the evolution of pollination ecotypes in the Cape shrub *Erica plukenetii*? Annals of Botany 113, 301-315.

Verboom, G. A., Archibald, J. K., Bakker, F. T., Bellstedt, D. U., Conrad, F., Dreyer, L. L., Forest, F., Galley, C., Goldblatt, P., Henning, J. F., 2009. Origin and diversification of the Greater Cape flora: ancient species repository, hot-bed of recent radiation, or both? Molecular Phylogenetics and Evolution 51, 44-53.

Waser, N. M., Campbell, D. R. 2004. Ecological speciation in flowering plants. Pages 264-277.*in* U. Dieckmann, Doebeli, M., Metz, J. A. J., Tautz, D., eds. Adaptive speciation. Cambridge University Press, Cambridge.

Waser, N. M., Price, M. V. 1993. Specialisation and generalisation in pollination systems. Pages 165-169 *in* International Symposium on Pollination in Tropics. IUSSI - Indian Chapter, Bangalore, India.

Wu, Z.-Y., Milne, R. I., Chen, C.-J., Liu, J., Wang, H., Li, D.-Z., 2015. Ancestral state reconstruction reveals rampant homoplasy of diagnostic morphological characters in Urticaceae, conflicting with current classification schemes. PloS one 10, e0141821.

CHAPTER 2 : A MORPHOLOGICAL AND PHYLOGENETIC STUDY OF THE *SATYRIUM NEGLECTUM* COMPLEX

2.1 Abstract

Classical taxonomy relies almost exclusively on the morphology of herbarium specimens to delimit species boundaries. Although herbarium records provide a means for both comparative morphological and geographical analyses, phylogenetic analyses of DNA sequences can be used in the assessment of perceived intraspecific variation between populations currently considered the same taxonomic species. *Satyrium neglectum* Schltr. is a highly variable taxon currently comprising of two subspecies, ssp. *neglectum* and ssp. *woodii*, both of which are widely distributed in subtropical southern and eastern Africa. Preliminary phylogenetic evidence and variation in morphology, both between the subspecies and among previous synonyms, as well as within ssp. *neglectum*, necessitates a re-evaluation of the systematics of the complex. A principal components analysis using traits measured from herbarium records from across the range, and ethanol-preserved flowers from South Africa, confirmed the presence of two clusters within ssp. *neglectum*, separating specimens from high elevation sites in the South African Drakensberg from the rest. Records of the synonym var. *brevicalcar*, previously considered to be similar to the high elevation form, are closer in quantitative trait space to the other specimens of *S. neglectum* ssp. *neglectum* and can be distinguished by several features from the South African high-elevation form in herbarium specimens. Ethanol-preserved flowers of ssp. *woodii* are distinct in multivariate trait space when compared to those of South African representatives of ssp. *neglectum*, but shares overlap if analysed when herbarium records of the concept are compared together with its synonyms *S. sceptrum* and *S. acutirostrum* from across the range. Despite incongruence between topologies derived from plastid and nuclear DNA sequence data, Bayesian inference from DNA sequences, including accessions sampled from an expanded geographic range, revealed strong support for monophyly of the South African high-elevation form of ssp. *neglectum*. Accessions of the remaining accessions of ssp. *neglectum* and ssp. *woodii* are, however, not reciprocally monophyletic. Although the distinctiveness of the high-elevation form in the phylogenetic analyses is not entirely matched by quantitative analyses of traits from herbarium records, cumulative evidence suggests that the variation between the South African high-elevation form and the remainder of ssp. *neglectum* warrants recognition of two independent species.

Consistent with previous taxonomic analyses, ssp. *neglectum* and ssp. *woodii* are best recognized as subspecies.

Keywords: *Orchidaceae, morphology, phylogeny, multivariate analysis, Satyrium, Bayesian analysis, ITS, plastid*

2.2 Introduction

Accurate species-level taxonomy is not only relevant for science, but also vital for conservation. Taxonomy prior to the mid-20th century relied heavily on the use of morphology for taxonomic delimitation (Dressler, 1993; Raven, 2004; Chase, 2005; Sosef et al., 2020). However, results from systematic studies based on molecular data have shown that although morphological characters may often be indicative of phylogenetic relationships, numerous traits considered to be important for species delimitation are homoplasious (Freudenstein and Rasmussen, 1999; Chase, 2005). Similar traits that have evolved multiple times through adaptation to a specific environment (convergence) can be particularly problematic if used for species delimitations and for phylogenetic reconstruction. The exclusive use of morphology in taxonomy is therefore problematic due to the possibility of erroneous implementation of homoplasious diagnostic traits and, in the case of the exclusive use of herbarium specimens, lack of available material or traits due to pressing (Parnell et al., 2013; Botes et al., 2020). Conversely, molecular phylogenetics that is not informed by accurate species delimitation based on morphology can be misleading and result in artificial groupings of recently diverged taxa that have very similar or even identical sequences, yet differ in key features. Multidisciplinary studies which combine assessment of herbarium specimens and phylogenetic analyses may reveal that species analysed by morphology alone may consist of multiple taxa or lead to resurrection of concepts which were previously relegated to synonymy (Hodges and Arnold, 1994; Botes et al., 2020).

Satyrium neglectum Schltr. is a species that is highly variable in morphology, and is distributed from the Eastern Cape province of South Africa, into Lesotho, Zimbabwe and Mozambique, with an extension into countries closer to the equator such as Kenya, the Democratic Republic of the Congo, Malawi and Tanzania (Moriarty, 1975; Hall, 1982; la Croix, 1991). The current taxonomic classification of *Satyrium neglectum* is based on the most recent revision of the genus (Hall, 1982), in which two subspecies were recognized: *S. neglectum* ssp. *woodii* (Schltr.) A.V. Hall and *S. neglectum* ssp. *neglectum* (Schltr.) A.V. Hall (Figure 2.1).

Historically, the species has undergone numerous taxonomic changes and various taxa have been incorporated into the current concept and were henceforth considered to be synonymous (Table 2.1).

Following the description by various authors of several species with a relatively narrow distribution, Summerhayes (1966) was the first to revise the taxonomy of the *Satyrium neglectum* species by relegating *S. densum* (Rolfe, 1898) and *S. colliferum* (Schlechter, 1915) as synonyms of *S. neglectum*. Based on examination of records of *S. neglectum* from Tanzania, Summerhayes (1966) did, however, find that a new variety needed to be described: *Satyrium neglectum* var. *brevicalcar*. This concept was based on records located predominantly in South-Central Africa, most notably in areas of particularly high elevation in Malawi and Tanzania, and included the addition of *Satyrium papyretorum* (Schlechter, 1916) as a synonym within var. *brevicalcar*. Further changes to the taxonomy of *Satyrium neglectum* and all related taxa occurred in Hall (1982), based on a quantitative analysis of traits measured on herbarium specimens. Hall's *Satyrium neglectum* ssp. *neglectum* includes the combination of the concepts *S. neglectum* var. *neglectum* and the short-spurred variety *Satyrium neglectum* var. *brevicalcar* (Summerhayes, 1966; Hall, 1982). Hall (1982) did suggest the existence of two forms of *S. neglectum* ssp. *neglectum*. The most common form is distributed at relatively low elevation and is characterised by having pink flowers, occurring commonly in the foot-hills of the Drakensberg mountains, but has also been found to occur throughout Zimbabwe, Mozambique, Malawi and Tanzania, (Hall, 1982; la Croix, 1991; Pope, 1995). A rarer form is distributed in areas of high elevation, with particular reference to the high slopes of the Drakensberg range in southern Africa, and is characterised by having pale white-cream flowers, with purple tinges along the middle of the sepals and petals. The previous description of var. *brevicalcar* was explicitly linked to the high-elevation form, and the two were considered to be the same by Hall (Hall, 1982).

The second subspecies recognized by Hall (1982) was ssp. *woodii*, which Hall (1982) considered a combination of the concepts of *Satyrium woodii* (Schlechter, 1895), *Satyrium beyrichianum* (Kraenzlin, 1898), *Satyrium sceptrum* (Schlechter, 1915), and *Satyrium acutirostrum* (Summerhayes, 1931), all of which have in some way been considered morphologically similar to *S. neglectum* by their original authors. These concepts were considered not adequately distinguishable and therefore relegated to synonymy by Hall (1982). The ssp. *woodii* concept is described as having large, relatively few long-spurred flowers,

which are arranged on a robust inflorescence and varying in colour, from brick-red, orange to sometimes pink (Hall, 1982). Despite its inclusion in ssp. *woodii*, *S. sceptrum* is often still considered a distinct and separate entity, being readily recognised and distinguished from *S. woodii* in the field, owing to differences in both flower colour, and the curvature of sepals and petals, leading to some contention regarding its taxonomic placement (Pope, 1995; Van der Niet et al., 2005; Van der Niet and Linder, 2008).

The taxonomy of the *S. neglectum* complex proposed by Hall (1982) has several implications. Due to the wide distribution of both subspecies, they are of low conservation priority and a potential subdivision of some of the entities within the species complex might change this. Understanding of the evolution of the species complex also requires well-resolved taxonomy. For instance, studies of the pollination ecology of populations of *S. neglectum* in South Africa suggest the presence of at least three specialized pollination systems, including birds, butterflies and long-proboscid flies, in ssp. *woodii* and ssp. *neglectum* respectively (Johnson et al., 2011; Johnson and Van der Niet, 2019). If the current taxonomy reflects phylogenetic relationships, this would mean that rapid pollinator shifts would have occurred. A reassessment of species boundaries, especially using phylogenetic information, may therefore be appropriate. This particularly applies to two components of Hall's (1982) taxonomy: the decision to combine the *S. woodii* complex and *S. neglectum* complex into a single species, and the decision to not recognize the South African high-elevation form of *S. neglectum*. Indeed, the description of the South African high-elevation form scarcely matches that of var. *brevicalcar*, with the two differing in floral colour, and the only similarity being distinctly shorter spurs as compared to the low-elevation form or ssp. *woodii*. However, it is known that spur length is a highly variable trait, and therefore often homoplasious (Anderson et al., 2014) In his quantitative analysis of variation in the *S. neglectum* complex Hall (1982) included several floral and vegetative traits, but Hall (1982) did not explicitly include the geographical origin of the specimens, which precludes and limits the application of a geographical perspective to morphological variation.

Preliminary evidence from a species-level phylogenetic study of *Satyrium* found that southern African accessions collected from populations of the high-elevation form from the Drakensberg Mountain range and accessions of ssp. *neglectum* and *S. sceptrum* collected from Kenya, Tanzania and Malawi did not form a monophyletic clade, and were instead separated, contrary to their taxonomic grouping (Figure 1. of Van der Niet and Linder, 2008). The non-monophyly

of *S. neglectum* provided evidence consistent with the existence of at least two species within ssp. *neglectum,* further suggesting that the South African high-elevation form might constitute an independent species. Alternatively, the large geographic distance between the areas from which *S. neglectum* accessions were sampled may explain the non-monophyly, even if the taxa are conspecific (Van der Niet and Linder, 2008): a large geographical disjunction may result in reduced gene flow, and can therefore lead to genetic variation between geographically separate populations (Ferguson et al., 1998; Barbour et al., 2009; Britton et al., 2014).

The aim of this chapter was to investigate the species boundaries within the *Satyrium neglectum* complex using a combination of multivariate morphological and phylogenetic analyses, and additional information from herbarium collections. I implemented an expanded geographical sampling for molecular phylogenetic analyses to examine the phylogenetic relationships in the *S. neglectum* species complex. This was accompanied by an investigation of the morphology, with a specific focus on the comparison of specimens from different elevations and on the synonyms, which comprise the current concepts of both ssp. *neglectum* and ssp. *woodii*.

2.3 Materials and methods

2.3.1 Study Sites

Nine sites were visited for the acquisition of material for both the phylogenetic and morphological analyses. Sampling covered both high and low elevation populations of *Satyrium neglectum* ssp. *neglectum* in South Africa, particularly along the Drakensberg Mountain Range between February-March 2018-2020.

The high elevation form was sampled from three populations: Mt. Aux Sources (28°44'56.0"S 28°53'05.0"E; 3000 metres; referred to as A), Sani Pass summit (29°35'09.0"S 29°17'17.6"E; 2874 metres; referred to as S) and Naudes Nek (30°43'52.6"S 28°08'18.9"E, 2578 metres; referred to as N). The low elevation form was sampled from populations from Graskop (24°56'05.7"S 30°48'22.6"E; ≥ 1600 metres; referred to as G), Van Reenen's Pass (28°23'08.0"S 29°23'55.0"E; ~2000 metres; referred to as V), Sani Pass base (29°36'11.4"S 29°20'20.4"E, 2025 metres), Garden Castle (29°44'41.3"S 29°12'30.4"E, 1850 metres; referred to as GC), Bushman's Nek (29°50'42.9"S 29°11'59.9"E; ~2000 metres; referred to as B), and Qacha's Nek (30°08'00.9"S 28°40'48.3"E, 2015 metres; referred to as Q).

2.3.2 Morphological Analysis

2.3.2.1 Herbarium specimens

Herbarium records of *S. neglectum* ssp. *neglectum* and *S. neglectum* ssp. *woodii,* including their previous synonyms, were sourced from the Bew's (NU), Pretoria (PRE), and Kew (K) Herbaria, which account for records across the species complex range (Appendix A: Table S3). For the purpose of the analyses in this study each specimen was assigned a name. For ssp. *neglectum*, the main focal taxon of this study, this was based on visual inspection of the herbarium specimens and taxonomic opinion. In particular, specimens that were previously identified as var. *brevicalcar* sensu Summerhayes (1966), which are exclusively distributed outside South Africa, are referred to as 'var. *brevicalcar'*, even though Hall (1982) considered specimens from short-spurred high-elevation form in the Drakensberg Mountains to also belong to this taxon. Specimens from high elevation in the Drakensberg mountains in South Africa, which can be unambiguously identified based on the colouration of the perianth, stunted growth form, short leaves, and relatively long spreading sepals and petals, are referred to as 'high-elevation form'. All other specimens are referred to as 'low-elevation form', with a distinction between material of this form from South Africa, and all other countries from which collections were available. Specimens of ssp. *woodii* are referred to by the names given by previous taxonomists, which include *S. sceptrum*, *S. acutirostrum*, and *S. woodii*.

A total of 16 morphological traits (Table 2.4; Appendix A: Table S1) were measured on one plant per herbarium accession. Traits were measured using either digital callipers or a ruler with a 0.5 mm scale. Trait measurement focused on floral traits between different concepts, vegetative traits were limited to the vegetative stem. Leaves were often not included in records of the high-elevation form and could therefore not be measured. To compare variation in morphological characters as well as visualize differences within the species complex, a principal components analysis with a correlation matrix, which was calculated using standardized data by subtracting the mean and dividing by the standard deviation. An Analysis of Similarity (ANOSIM), with Euclidean distance, which was then converted into Euclidean similarity index in PAST (v.4) was used to test for differences between the concepts and synonyms within *S. neglectum* species complex.

To compare records and analyse differences in means of traits between specimens of var. *brevicalcar* from East Africa and the Drakensberg mountains of South Africa, as well as records from low-elevation, a generalised linear model with a normal distribution and identity link function was used. In the case of count data, such as the number of flowers, data were

analysed with a negative binomial distribution with a log link function was used instead. To correct for multiple comparisons, the sequential Šidák correction was used.

To examine if records of the high- and low-elevation form conform to distinct elevation groups, the distribution of herbarium records along an elevation gradient was recorded from the information on the specimen label, of South African records of the high- and low-elevation forms which was recorded on the collector sheet, or inferred if adequate description of locality was given. A frequency distribution was constructed from the elevation data. In addition, the distribution of specimens along elevation and morphology of the two forms was examined.

2.3.2.2 Ethanol-preserved flowers

Characters of herbarium specimens cannot always be accurately measured due to distortion caused by pressing or due to being concealed (Botes et al., 2020). Floral traits were therefore also scored from flowers collected from the 6 study sites of the high and low-elevation form (Mt. Aux Sources, Sani Pass summit, Naudes Nek, Van Reenen's Pass, Garden Castle and Bushman's Nek) and subsequently preserved in ethanol. Traits (Appendix A: Table S2) were measured from one flower per inflorescence, which were collected from the middle-to-lower third of the raceme to ensure flower maturity. Collected flowers were immediately stored in a mixture of 70% ethanol and a few drops of glycerol for pliability. Flowers were collected from populations of *Satyrium neglectum* ssp. *neglectum* and *S. neglectum* ssp. *woodii*. Flowers of populations of ssp. *neglectum* occurring at low-elevation were collected from Van-Reenen's Pass (n = 4), Garden Castle (n = 23) and Bushman's Nek (n = 15), whereas flowers from populations occurring at high-elevation were collected from Mt. Aux-Sources (n = 15), Sani Pass (n = 14) and Naude's Nek (n = 38). Flowers of *S. neglectum* ssp. *woodii* (n = 19) were collected from one of the few known sites for this species in South Africa (Johnson and Van der Niet, 2019).

Selection of traits for measurement in preserved flowers focused on the morphology of the gynostemium and included measurements of the length and width of the petals, sepals, apical flap, ovary, column, and the stigma flap (see Supplementary Table S2 for a detailed overview of all traits measured). The perianth of ethanol preserved flowers was carefully dissected and measured under a compound microscope. A principal components analysis with a correlation matrix was used to visualize differences between the high-, low-elevation forms and ssp. *woodii* forms, in addition to an Analysis of Similarity (ANOSIM) with Euclidean distance, which was converted into Euclidean similarity index in PAST (v.4).

To compare variation in mean traits between flowers of the high-, low-elevation form and ssp. *woodii* a Generalized Linear Model was used. To compensate for the lack of independence in flowers measured form the same locality, population was used as subject in generalized estimating equations, implementing an exchangeable correlation matrix and using a normal distribution and identity link function. To compensate for multiple comparisons, the sequential Šidák procedure was implemented. All univariate statistical analyses of morphological data were performed in SPSS v.27 (IBM Corps.), whereas multivariate analyses were performed in PAST v.3 (Hammer et al., 2001).

2.3.2.3 Scanning Electron Microscopy

To determine if there is a difference in the structure of the gynostemium and rostellum, both traits which are important in *Satyriinae* and orchid delimitation in general, flowers (n = 4; two from each form) of the high- and low-elevation form were sampled from Mt. Aux Sources and Bushman's Nek respectively and stored in 70% ethanol prior to analysis. Samples were prepared for scanning electron microscopy using the methods outlined in Van der Niet et al. (2011). The sepals, petals and galea were removed to reveal the gynostemium and stigma flap structure and then transferred to 100% ethanol. Samples were brought to critical drying point using CO_2 as a carrier gas. Samples were sputter coated using a mixture of gold and platinum. Scanning electron images were captured using an Zeiss Evo LS15 scanning electron microscope

2.3.3 Phylogenetic Analysis

To determine the phylogenetic relationship among populations of *S. neglectum* ssp. *neglectum,* and within the *Satyrium neglectum* complex as a whole, a phylogenetic analysis using DNA sequences as characters was performed. The expanded sampling complements the phylogenetic analysis by Van der Niet and Linder (2008).

2.3.3.1 Taxon sampling

Samples of *S. neglectum* species complex included those of ssp. *neglectum* from the nine populations described above (see Table S4 for GenBank accession codes of these samples) as well as the samples of ssp. *neglectum* and ssp. *woodii* used in Van der Niet and Linder (2008). Outgroup sampling focuses on members closely related to the *S. neglectum* species complex (Van der Niet, 2017). This includes, *S. monadenum, S. buchananii,* and both varieties of *S. longicauda.* For rooting of the phylogenetic tree *Satyrium cristatum* was used for the plastid

analysis, whereas *Satyrium lupulinum* was used for ITS. These taxa were trimmed from the phylogenetic trees displayed here.

2.3.3.2 DNA Isolation, Amplification and Sequencing

Fresh leaf samples (2-3 leaves) were collected from 3-4 randomly selected individuals in each population and subsequently dried in silica gel after collection. DNA was extracted using the Dneasy Plant Mini Kit (Qiagen, Basel, Switzerland) following the manufacturers protocol. PCR mixtures contained: 1µl of DNA template, 12.5µl of OneTaq Master Mix, 1µl of bovine serum (BSA), 0.5 µl of forward primer (10 mM) and 0.5µl of reverse primer (10 mM), and 9.5 µl pure water up to a total volume 25µl.

The complete Internal Transcribed Spacer (hereafter referred to as "ITS") was amplified using the primer pair ITS 4 and ITS 5 (White et al., 1990). Thermocycling conditions for ITS were as follows: first an initial denaturation cycle for 30 seconds at 94˚C was implemented, followed by 35 cycles of 1-minute denaturation at 94˚C, 30 seconds annealing at 55˚C, and 1-minute extension at 72˚C, followed by final extension for 4 minutes at 72˚C.

The entire coding region of matK and parts of the trnK intron (hereafter referred to as "matK") were amplified with a combination of the external primer pair -19F (Kores et al., 2000) and R1 (Kocyan et al., 2004), as well as the internal primers 580F and 1082F (Kocyan et al., 2004). The complete trnS-G intergenic spacer (hereafter referred to as "trnSG") was amplified using the primer pair trnS and trnG (Hamilton, 1999). Thermocycling conditions for both matK and trnSG were as follows: an initial denaturation at 94 ˚C for 30 seconds was implemented, followed by 35 cycles of 30 seconds denaturation at 94 ˚C, 1 minute at 52 ˚C, and 1 minute at 68 ˚C. This was then terminated with final extension at 68 ˚C for 5 minutes.

Finally, the trnL intron and trnL-trnF intergenic spacer (hereafter referred to as "trnL-F") was amplified using the primer pair f (Taberlet et al., 1991) and C3 (Van der Niet et al., 2005) Thermocycling conditions for trnL-F were as follows: an initial denaturation at 94°C for 30 seconds, followed by 35 cycles denaturation at 94 ˚C for 30 seconds. This was then followed by annealing for 1 minute at 55 ˚C and extension for 1 minute at 68 ˚C, which was then terminated by a final extension at 68 ˚C for 5 minutes.

PCR products were visualised by electrophoresis on 2% agarose gel in 1 × TBE buffer with GelRed nucleic acid gel stain (Biotium Inc., Fremont, CA, USA), using the manufacturer's protocol. Post-PCR clean-up and sequencing were performed at the Stellenbosch University Central Analytical Facility (South Africa), using the same primers as used in the PCR. In

addition, the matK samples were sequenced with the aid of additional internal primer pair: 580F and 1082F (Kocyan et al., 2004). Forward and reverse sequences were assembled into contigs using the Geneious software v.11.15 prior to analysis. Sequences were compared and aligned by eye with previously published DNA templates from the genus, this included sequences of ssp. *neglectum* and further *Satyrium* species, including *S. monadenum, S. buchananii*, and both varieties of *S. longicauda*, among others.

2.3.3.3 Bayesian Inference

Bayesian Inference (Huelsenbeck and Ronquist, 2001) was performed using the software MrBayes v3.2 (Ronquist and Huelsenbeck, 2003). Due to previously detected incongruence involving the *S. neglectum* species complex, the alignments were split and analysed as two separate plastid and nuclear datasets. Partitions in the plastid dataset included the application of the Gamma model for introns, spacer and 1st codon positions, while a combination of the 2nd and 3rd codon using Invgamma model. The optimal model of sequence evolution for ITS was found to be Gamma. Thus, ITS was analysed alone, while the plastid data set combined trnL-F, trnSG and matK. The optimal model for sequence evolution was calculated using the Akaike Information Criterion (AIC) as implemented in Modeltest 3.7 (Posada and Crandall, 1998). Five million generations were run with a sample taken every 5000 generations and with the first 200 samples set as burnin. The analyses reached convergence after five million generations and the sample size exceeded the minimum value as examined in Tracer 1.7.1 (Rambaut et al., 2018).

2.4 Results

2.4.1 Morphological Analysis

2.4.1.1 Herbarium Specimens

The quantitative analysis of morphological traits of herbarium records reveals that high-elevation specimens form a distinct cluster, if analysed with all records together, and with records from ssp. *neglectum* alone (Figure 2.2 & Figure 2.3). There is no separation within the remaining specimens of ssp. *neglectum* and between ssp. *neglectum* and ssp. *woodii* (Figure 2.2). Factors did not contribute equally, however floral size, including sepal and petal length mostly indicated differences between clusters (Table 2.3).

A multivariate comparison between the morphology of var. *brevicalcar* and the southern African high-elevation form shows limited overlap between the two taxa (Figure 2.3& Figure 2.4). Instead, specimens of var. *brevicalcar* share more overlap with the remaining specimens of the low-elevation form (Figure 2.3). The results of the ANOSIM ($p = 0.0001$; $R = 0.2893$) reveal that the high-elevation form and South African records of the low-elevation form are significantly different from var. *brevicalcar* when compared using uncorrected p-values, although the difference between the two forms and var. *brevicalcar* becomes marginally non-significant following Bonferroni Correction (Table 2.2). Comparison of specific traits between var. *brevicalcar* and both the high- and low-elevation form illustrates that various traits differ significantly between both var. *brevicalcar*, and the two forms (Table 2.4).

Considering just South African records, the high- and low-elevation form are significantly different from each other (Table 2.2). The distinctiveness between the high- and low-elevation forms within South Africa is further supported by a distinction in elevation, samples of the low-elevation form are found below 2200 m, whereas most samples of the high-elevation form are from 2200 - 2500 metres (Figure 2.5). When morphology is incorporated in a 3-Dimensional plot in combination with elevation, the separation between both forms through both elevation, and morphology is evident (Figure 2.6). Univariate comparison of traits finds that majority of the traits between the high- and low-elevation form are significantly different, with the exception of the width of petals and sepals, as well as lip flap ratio (Table 2.4).

Eastern African records of ssp. *neglectum* form a bridge between southern African records of ssp. *neglectum* and ssp. *woodii* in the multivariate analysis (Figure 2.2). Examination of the

distribution of ssp. *woodii* shows that southern African *S. woodii* records are more closely grouped with records of *S. acutirostrum*, whereas records of *S. sceptrum* fall in between ssp. *neglectum* and S. *woodii* and *S. acutirostrum* (Figure 2.2). However, when analysed alone, *S. woodii*, *S. acutirostrum*, and *S. sceptrum* do not form distinct clusters (Figure 2.7). Based on the ANOSIM the synonyms that constitute ssp. *woodii* do not differ from each other, nor from low-elevation records of ssp. *neglectum* collected from East Africa (Table 2.2)

2.4.1.2 Analysis of Ethanol-Preserved specimens

Quantitative analysis of characters measured from ethanol-preserved flowers of South African records of the *S. neglectum* species complex reveals a clear distinction within the complex. Records from the three groups are all significantly different from one another through an Analysis of Similarity ($p = 0.0001$; Bonferroni corrected p-value = 0.0003). When comparing the three groups through principal components analysis, clusters representing the high- and low-elevation forms of ssp. *neglectum* as well as ssp. *woodii* are all clearly defined, with minimal overlap (Figure 2.8). In addition, a generalised linear model found that all traits with the exception of one are significantly different between all groups (Table 2.6), with some differentiation between traits related to the sepals, petals, column and rostellum (Figure 2.9). Axes loadings of characters do not contribute equally to differences between clusters (Table 2.5). Traits which contributed most to separation included: spur length, lengths of the median sepal and lateral sepal as well as the length of the labellum lip and column (Table 2.5).

In addition, the flowering times for the two forms overlap for the most part. Populations of the low-elevation form were found well into the middle of March in Graskop, Mpumalanga, while populations of the high-elevation form at Naudes Nek were mostly finished flowering towards the end of February. It seems the high-elevation form has a relatively short flowering time (Late Jan – Mid/Late Feb), whereas the low-elevation form flowers for a longer period (Jan to Mid-March; possibly longer as some individuals were only partly finished flowering).

Scanning electron microscopy images of the gynostemium of individuals from high- and low-elevation populations from South Africa illustrate differences in the structure of the rostellum (Figure 2.10). The rostellum of the high-elevation form has a much sharper, pointed apex when compared to the low-elevation form, which has a rostellum beak which is more flared, with a pointed apex (Figure 2.10). The viscidia of the high-elevation form, are rounder then those found in the low-elevation form, though both have a slightly plate-like appearance.

2.4.2 Phylogenetic Analysis

Accessions from South African populations at high-elevation are strongly supported as a monophyletic clade that is separate from both remaining ssp. *neglectum* accessions and ssp. *woodii* accessions (PP for both plastid and ITS = 1). The placement of South African accessions from low elevation and ssp. *woodii* differ between the plastid and nuclear data sets (Figure 2.11). The topology based on the plastid data illustrates a strongly supported clade comprised of South African accessions of ssp. *neglectum* from the low-elevation form with ssp. *woodii* (PP of plastid = 0.99) nested inside it, sister to an accession of *S. longicauda* var. *jacottetianum* (PP of plastid = 0.97). Accessions of ssp. *neglectum* from outside South Africa form a clade with the former synonym *S. sceptrum* from Kenya, Tanzania and Malawi, as well as *S. buchananii* (PP in plastid = 0.95; Figure 2.11). In the ITS topology, South African accessions from low-elevation also form a strongly supported clade with a South African accession of ssp. *woodii* (PP of ITS = 0.99). This clade excludes accessions of *S. longicauda* var. *jacottetianum*. The separation of South African and Eastern African accessions of the low-elevation form and *S. sceptrum* and ssp. *woodii* is also retrieved in the ITS dataset, which for the most part forms a strongly supported trichotomy (PP of ITS = 1; Figure 2.11), with one accession of *S. neglectum* from Mulanje being unresolved. All accessions of the *S. neglectum* species complex are sister to *S. monadenum*, and together this clade is sister to a clade comprising *S. longicauda*. The high elevation form is sister to this overall combined clade (Figure 2.11).

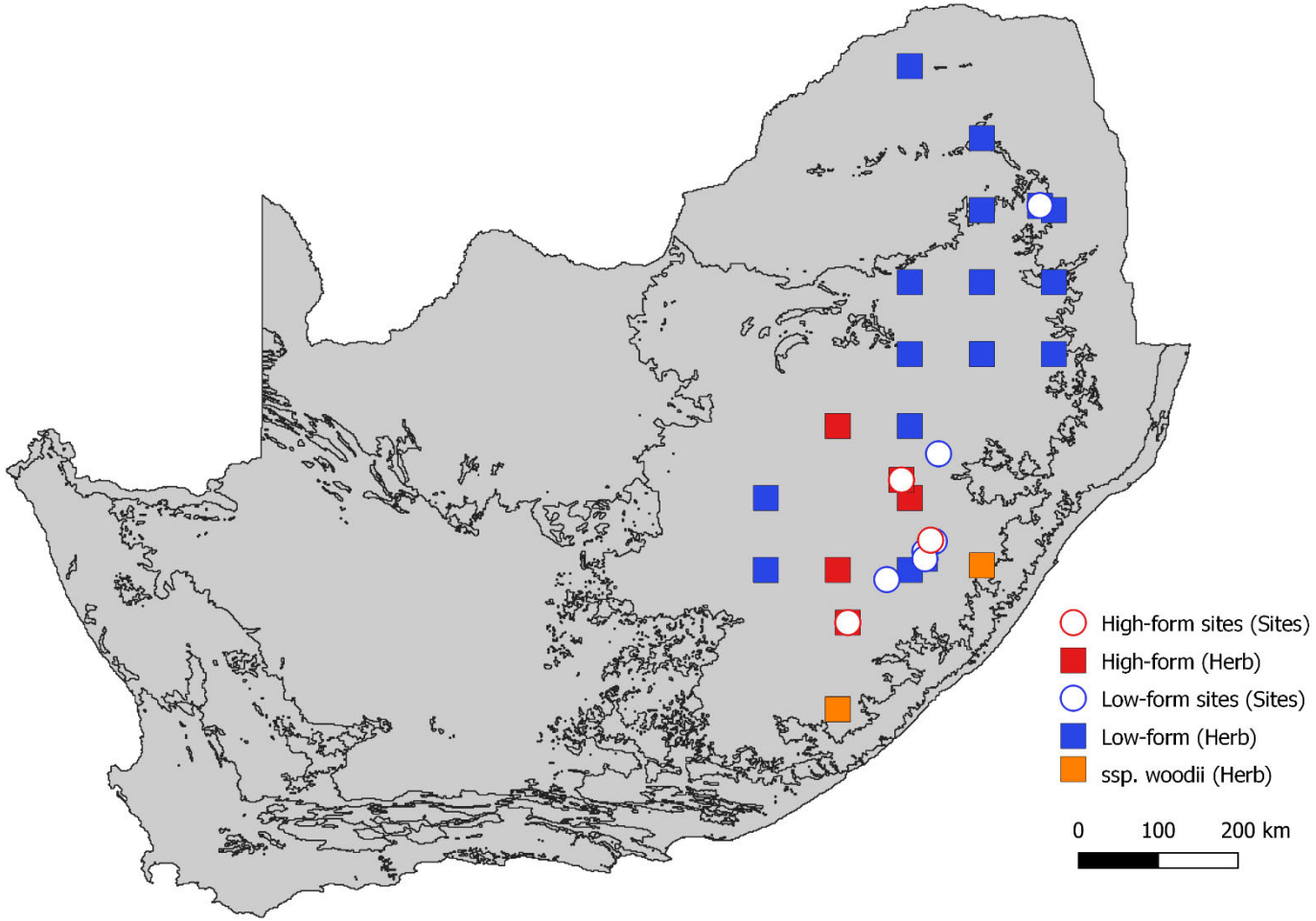

Figure 2.1: Distribution of the *Satyrium neglectum* species complex in South Africa based on herbarium collections from KEW, PRE, NU.

Table 2.1: Synonyms of the *Satyrium neglectum* species complex as defined in Hall (1982).

Species	Author (year)	Current Classification	Locality	Defining Traits
S. neglectum	Schlechter (1895)	ssp. *neglectum*	South Africa	Flowers small, pink with highly retracted sepals and petals, densely packed on inflorescence.
S. woodii		ssp. *woodii*	South Africa	Flowers robust, often orange. sometimes brick-red, pink.
S. densum	Rolfe (1898)	ssp. *neglectum*	Malawi	Trilobed rostellum, with a pointed, spathulate frontal lobe arising from the middle of two-minute lateral tooth-shaped lobes
S. beyrichianum	Kraenzlin (1898)	ssp. *woodii*	South Africa	Similar to *S. longicauda*. Described as having much larger flowers.
S. colliferum	Schlechter (1915)		Malawi	Described as a close relative to *S. neglectum* due to similar narrowing of upper part of ovary
S. sceptrum		ssp. *woodii*	Tanzania	Flowers are yellow on the inside, progressing to brick-red on the outside. Spurs as long as the ovary. Stem purple on upper part of inflorescence, green towards the base.
S. papyretorum	Schlechter (1916)	ssp. *neglectum*	Zimbabwe	Similar to var. *brevicalcar* due placement of lateral leaf shoots and overall similar morphology.
S acutirostrum	Summerhayes (1931)	ssp. *woodii*	Belgian Congo	Considered affiliated to *S. sceptrum*, distinguished by larger flowers, spurs, intermediate pointed rostellum lobe
var. *neglectum*	Summerhayes (1966)	ssp. *neglectum*	South Africa	Flowers small, pink, highly retracted sepals and petals, densely packed on inflorescence.
var. *brevicalcar*		ssp. *neglectum*	Malawi and Tanzania	Yellow flowers with brown streaks and distinctly short spurs.
ssp. *neglectum*	Hall (1982)		Extensive	Flowers often pink, rarely, white-cream with purple tinges. Petals and sepals most often retracted. Spur length varies considerably.
ssp. *woodii*				Few flowers, orange, yellow, sometimes pink, rose-coloured on a robust, large inflorescence.

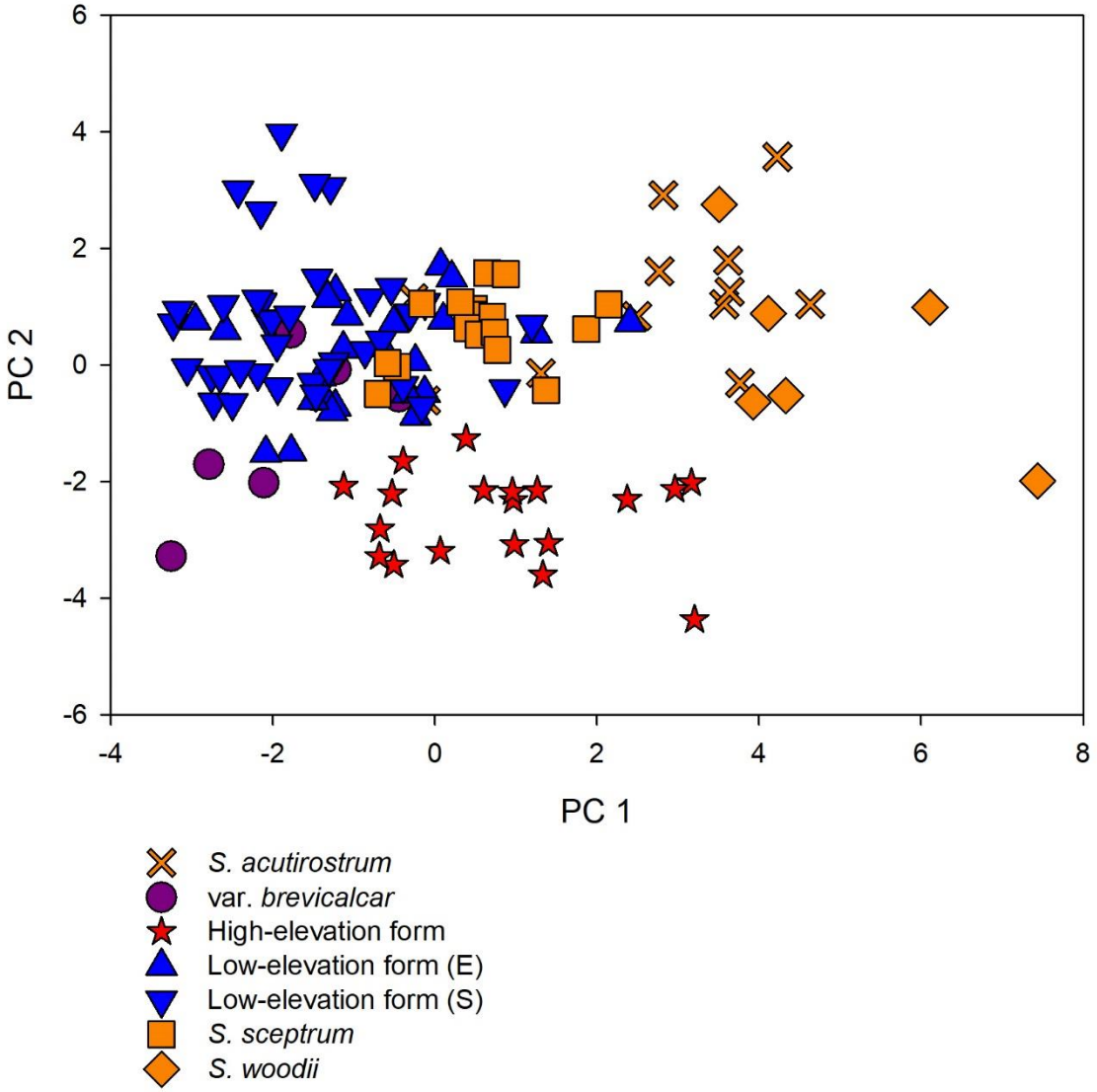

Figure 2.2: Ordination based on a Principal Components Analysis of 16 morphological characters of the *Satyrium neglectum* species complex throughout the species range in herbarium records. PC1 explains 28.85% of the variation, whereas PC2 explains 15.76% of the variation (Blue = low-form; red = high-form; purple = var. *brevicalcar*; orange = ssp. woodii records; S = South African/Lesotho; E = eastern African).

Table 2.2: Analysis of Similarity (ANOSIM) comparing synonyms of the S. *neglectum* species complex measured from herbarium records, based on 9999 permutations using Euclidean Similarity without/with Bonferroni Corrected p-values (S = South African/Lesotho; E = eastern African)

	S. acutirostrum	var. *brevicalcar*	High-form	Low-form (S)	Low-form (E)	*S. sceptrum*	*S. woodii*
S. acutirostrum		0.0761/1.000	**0.0001 / 0.0021**	**0.0001 / 0.0021**	0.4988 / 1	0.1714 / 1.000	0.903 / 1.000
var. *brevicalcar*	0.0761 / 1.000		**0.0036 /** 0.084	**0.003 /** 0.063	0.8932 / 1	0.7731 / 1.000	**0.039 /** 1.000
High-form	**0.00011 / 0.0028**	**0.0036 /** 0.084		**0.0001 / 0.0021**	**0.0002 / 0.0063**	**0.0001 / 0.0063**	**0.0001 / 0.0042**
Low-form (S)	**0.0001 / 0.0028**	**0.003 / 0.0448**	**0.0001 / 0.0021**		**0.0001 / 0.0021**	**0.0001 / 0.0021**	**0.0008 / 0.0084**
Low-form (E)	0.4988 / 1.000	0.8932 / 1.000	**0.0002 / 0.0063**	**0.0001 / 0.0021**		0.7946 / 1.000	0.6949 / 1.000
S. sceptrum	0.1714 / 1.000	0.7731 / 1.000	**0.0001 / 0.0021**	**0.0001 / 0.0021**	0.7946 / 1.000		0.43 / 1.000
S. woodii	0.903 / 1.000	**0.039 /** 0.8001	**0.0001 / 0.0042**	**0.0008 / 0.0084**	0.6949 / 1.000	0.43 / 1.000	

Table 2.3: Character Loadings for Ordination based on a Principal Components Analysis of 16 morphological characters of the *Satyrium neglectum* species complex throughout the species range in Herbarium records (Numbers in bold indicate > 0.2 relationship)

Trait	PC 1 (28.5%)	PC 2 (15.76%)	PC 3 (14.15%)	PC 4 (9.18%)
Inflorescence Length	**-0.2012**	**0.31642**	**0.38128**	-0.14547
Flowering Stem Length	0.10927	**0.38777**	**0.22273**	-0.12599
Number of Flowers	**-0.21331**	**0.28137**	**0.38339**	-0.17697
Spur Length	0.091257	**0.32832**	**0.28583**	-0.11836
Median Sepal Length	**0.36297**	-0.10195	0.16804	-0.2652
Median Sepal Width	**0.24626**	**0.24714**	**-0.25667**	0.16594
Median Sepal Ratio	**0.21055**	**-0.27921**	**0.28838**	**-0.32508**
Lateral Sepal Length	**0.40129**	-0.05054	0.15444	-0.00312
Lateral Sepal Width	**0.3524**	**0.2851**	-0.10252	-0.05328
Lateral Sepal Ratio	0.025624	**-0.37671**	**0.25626**	0.078927
Petal Length	**0.36905**	-0.04953	0.1443	0.1224
Petal Width	**0.3207**	**0.26438**	-0.17962	0.073986
Petal Ratio	0.045402	**-0.30898**	**0.30598**	0.047649
Lip Flap Length	**0.23069**	-0.01265	**0.21297**	**0.48468**
Lip Flap Width	**0.27355**	-0.10961	-0.10103	-0.20111
Lip Flap Ratio	-0.04462	0.085915	**0.31103**	**0.63791**

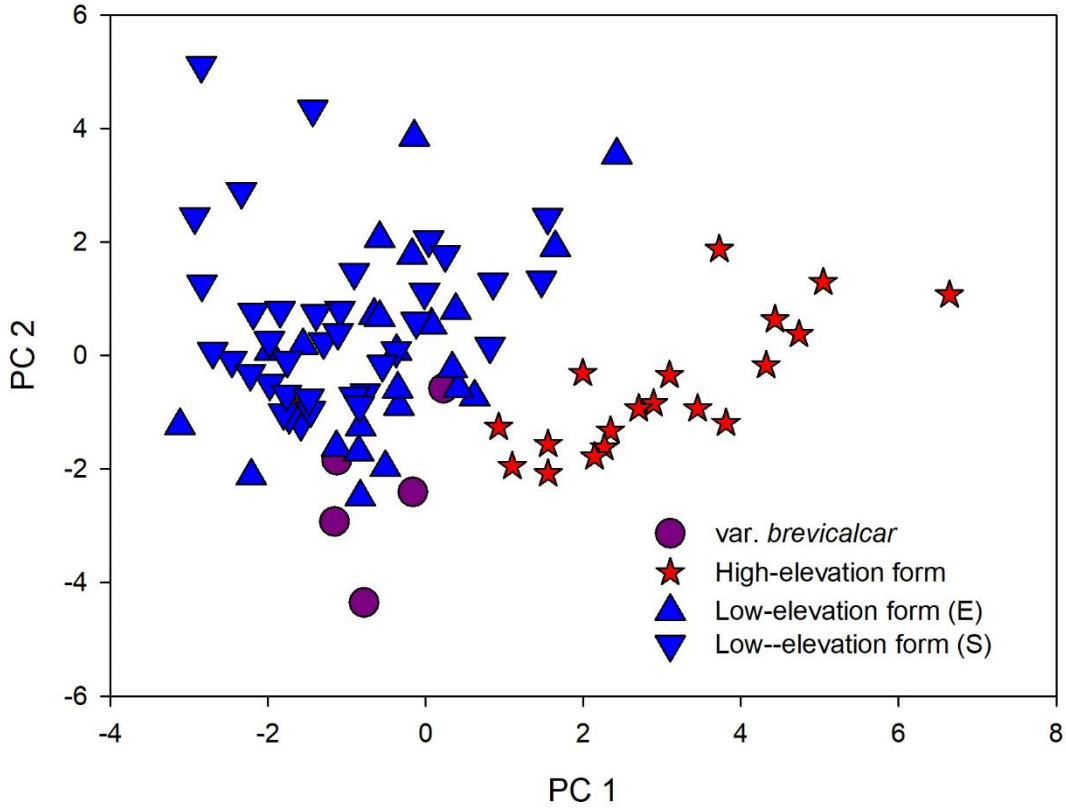

Figure 2.3: Ordination based on a Principal Components Analysis of the southern and eastern African herbarium records of *Satyrium neglectum ssp. neglectum* and the synonym *Satyrium* var. *brevicalcar* (Blue = low-form; red = high-form; purple = var. *brevicalcar*) where 26.1% of the variation is explained by PC 1, and 16.75% is explained by PC 2.

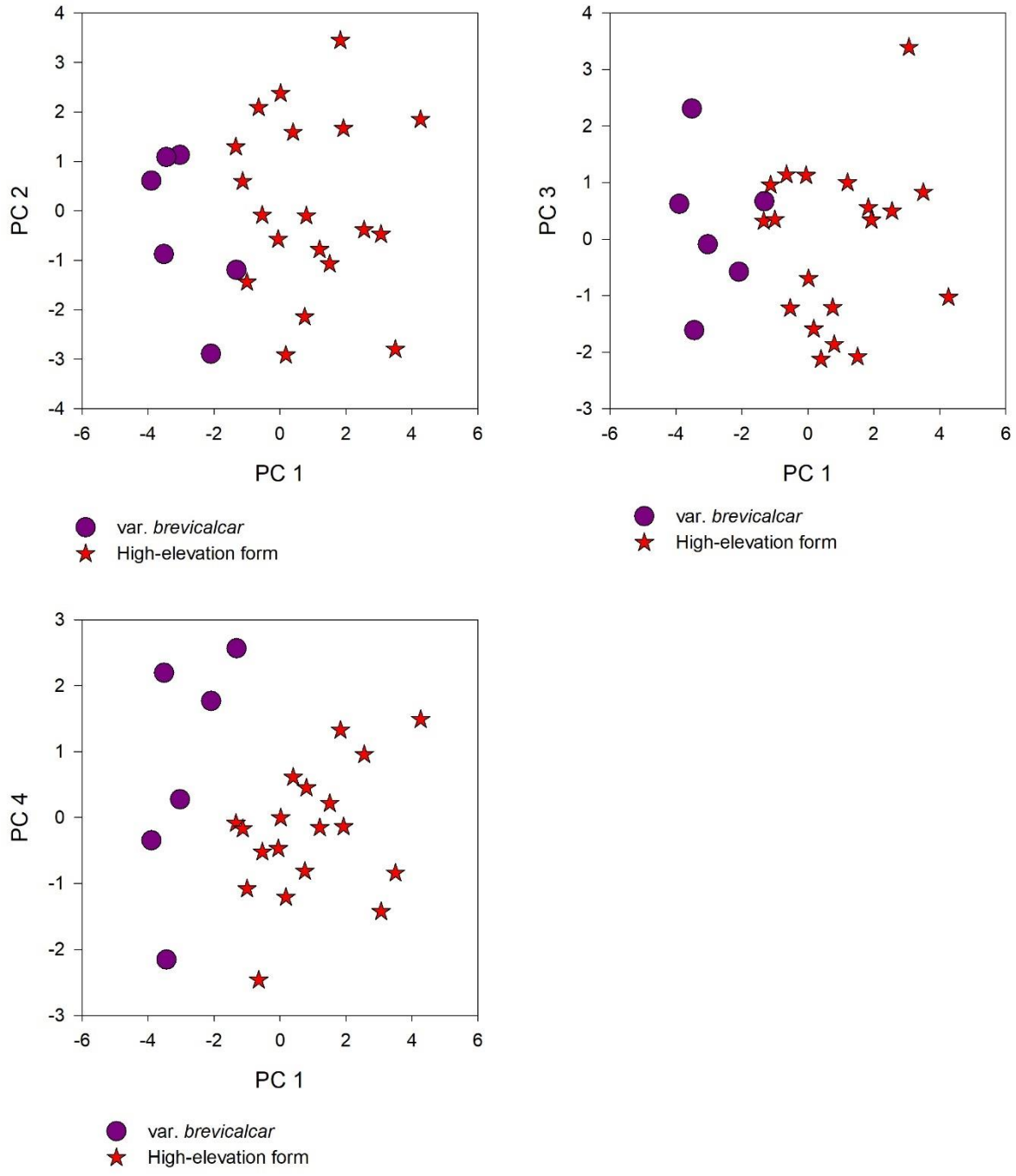

Figure 2.4: Ordination based on a Principal Components Analysis of the high-elevation form of *Satyrium neglectum* ssp. *neglectum* (red) and the synonym *Satyrium* var. *brevicalcar* (purple) where 64.64% of the variation is explained by PC 1, 29.9% is explained by PC 2, while 2.9% is explained by PC 3 and 1.63% is explained by PC 4.

Table 2.4: Mean ± SD of measured traits (all in cm) of the high-, low-elevation form and previous synonym, var. *brevicalcar* measured from herbarium records. Statistical significance between the means is: NS – non-significant, * P <0.05, ** P < 0.01, ***P < 0.001

Trait	**Mean ± SD**			**P** [2]		
	High-form	var. *brevicalcar*	Low-form	H vs. B	L vs. B	H vs. L
Inflorescence Length	9.27 ± 2.56	11.53 ± 2.71	19.57 ± 7.91	NS	***	***
Flowering Stem Length	16.71 ± 5.71	29.25 ± 12.17	39.19 ± 13.64	*	*	***
Number of Flowers[1]	21.22 ± 7.54	21.33 ± 7.28	44.39 ± 20.9	NS	NS	***
Spur Length	0.89 ± 0.16	0.88 ± 0.24	1.36 ± 0.24	NS	***	***
Median Sepal Length	0.61 ± 0.15	0.37 ± 0.07	0.42 ± 0.13	***	NS	***
Median Sepal Width	0.14 ± 0.05	0.11 ± 0.04	0.14 ± 0.04	NS	NS	NS
Median Sepal Ratio	4.67 ± 1.53	3.36 ± 0.6	3.03 ± 0.98	*	NS	***
Lateral Sepal Length	0.6 ± 0.09	0.41 ± 0.06	0.48 ± 0.09	***	NS	***
Lateral Sepal Width	0.18 ± 0.03	0.17 ± 0.04	0.19 ± 0.04	NS	NS	NS
Lateral Sepal Ratio	3.34 ± 0.58	2.55 ± 0.75	2.58 ± 0.65	*	NS	**
Petal Length	0.54 ± 0.11	0.35 ± 0.07	0.43 ± 0.09	***	NS	***
Petal Width	0.15 ± 0.03	0.13 ± 0.03	0.14 ± 0.03	NS	NS	NS
Petal Ratio	3.8 ± 1.17	2.69 ± 0.53	3.1 ± 0.75	*	NS	***
Lip Flap Length	0.19 ± 0.04	0.13 ± 0.03	0.15 ± 0.03	**	NS	*
Lip Flap Width	0.2 ± 0.05	0.16 ± 0.02	0.15 ± 0.03	NS	NS	***
Lip Flap Ratio	1 ± 0.4	0.82 ± 0.11	1.02 ± 0.25	NS	NS	NS

[1]Analysed with a negative binomial distribution with log link function

[2]H = High-form; L = Low-form; B = var. *brevicalcar*

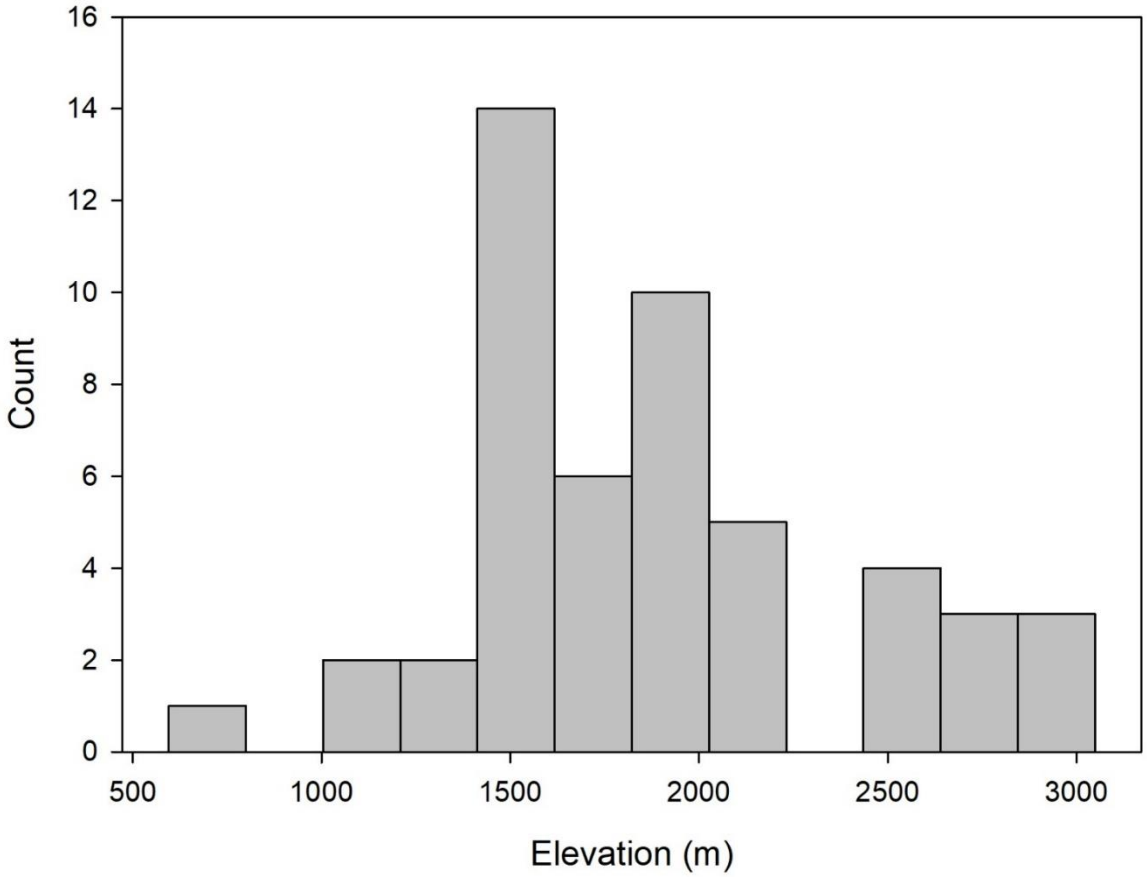

Figure 2.5: Frequency distribution of South African herbarium samples of *Satyrium neglectum ssp. neglectum* across the elevation range 500 - 3050 m (High-elevation form ≥ 2200 m; Low-elevation form ≤ 2135 m).

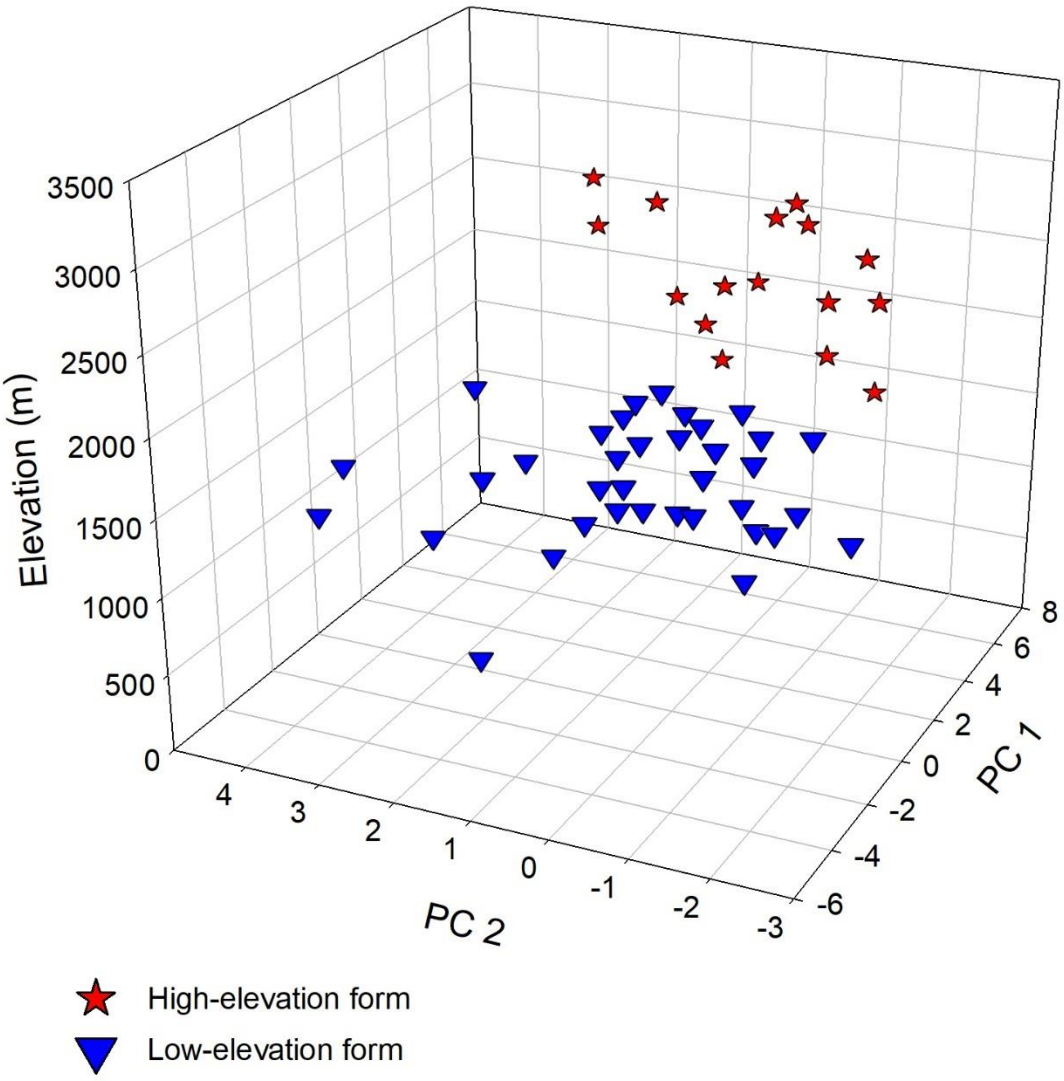

Figure 2.6: Principal Components Analysis Axes 1 and 2 against recorded elevation in herbarium samples of ssp. *neglectum* collected in South Africa.

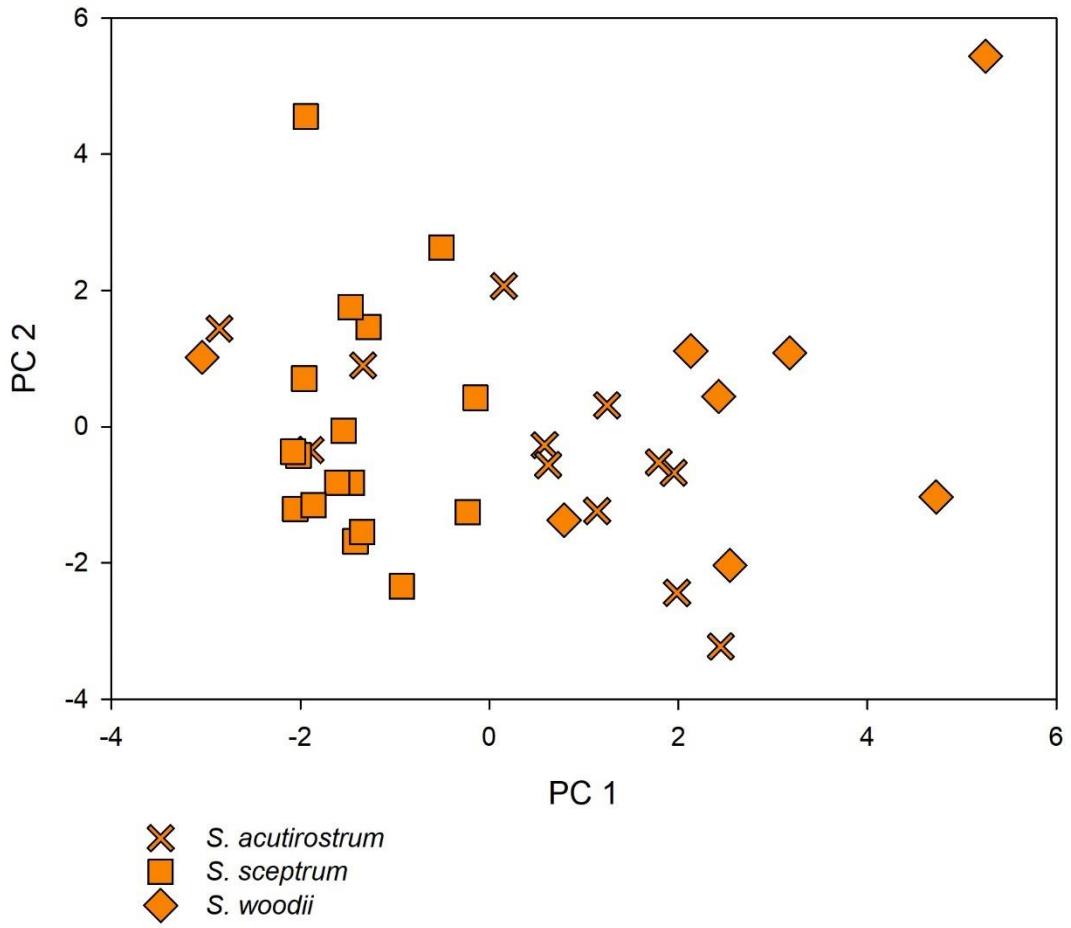

Figure 2.7: Ordination based on a Principal Components Analysis of the previous *S. acutirostrum*, *S. sceptrum* and *S. woodii*, where 27.24% of the variation is explained by PC 1, and 20.70% is explained by PC 2.

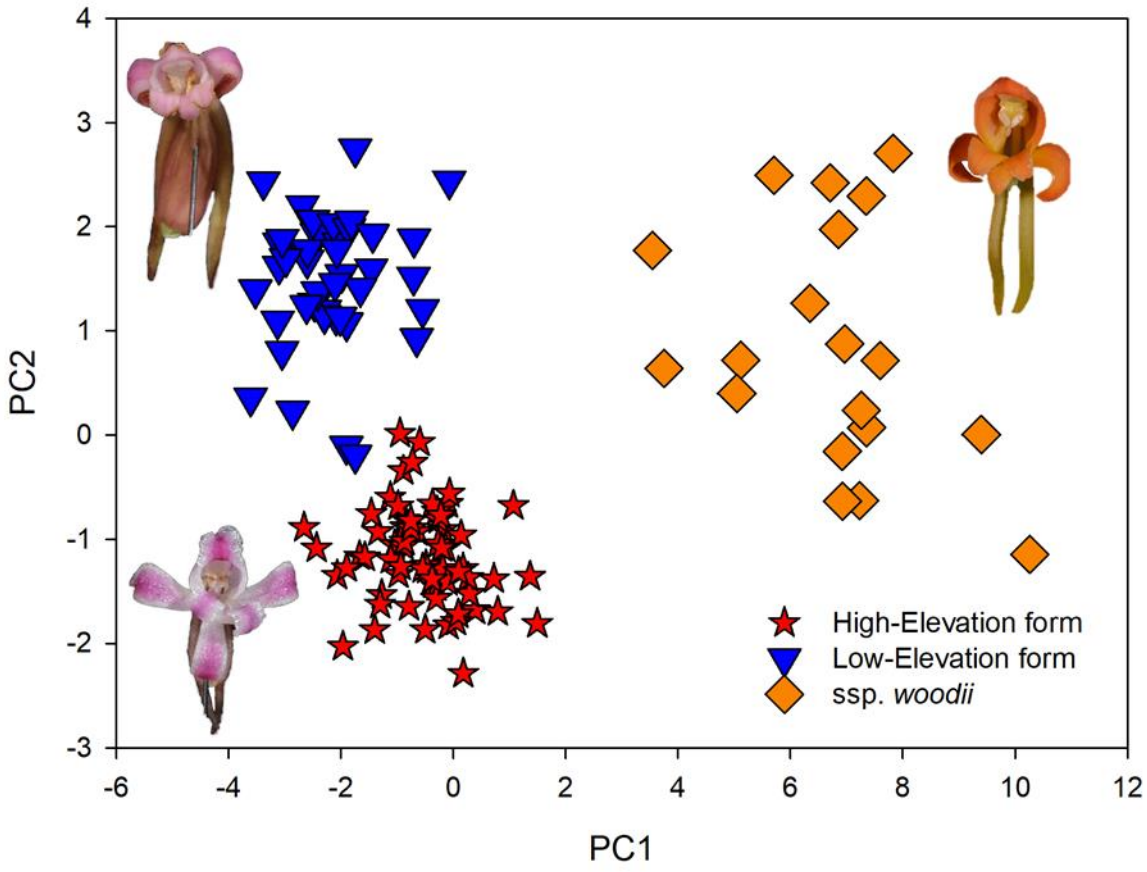

Figure 2.8: Ordination based on a Principal Components Analysis of 15 morphological traits measured from ethanol-preserved flowers from 7 sites of the high- (n = 67) and low-elevation forms (n = 42) of ssp. *neglectum* and ssp. *woodii* (n = 19) from South Africa, where 63.25% of the variation is explained by PC 1, and 13.26% is explained by PC 2.

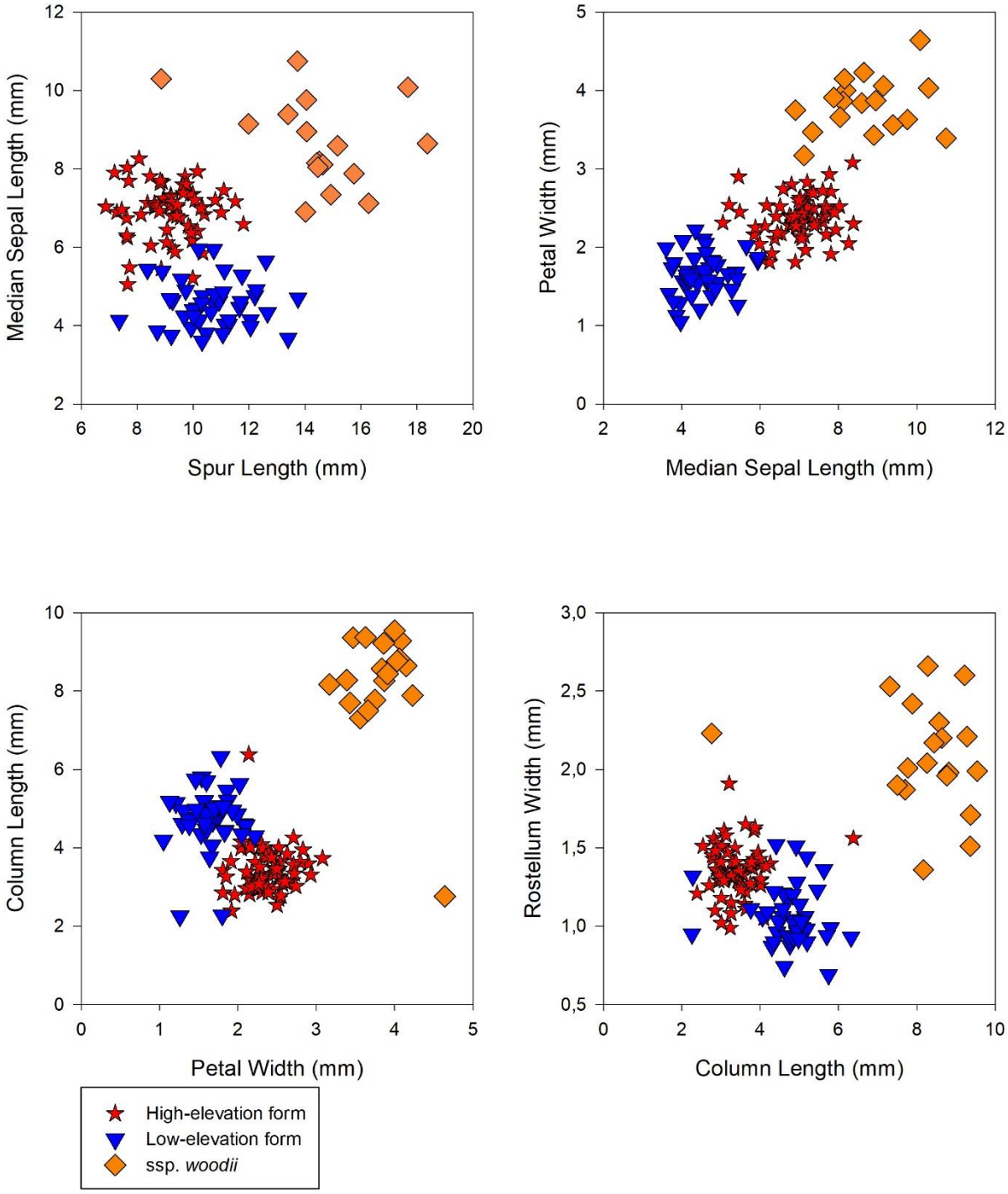

Figure 2.9: Scatter plots indicating differentiation in measurements in ethanol-flowers of the sepals, petals, spurs column and rostellum in *S. neglectum* species complex.

Table 2.5: Character Loadings for Ordination based on a Principal Components Analysis of 15 morphological characters of the *Satyrium neglectum* species complex throughout the species range in Ethanol flowers (Numbers in bold indicate > 0.2 contribution)

Trait	PC 1 (63.25%)	PC 2 (13.26%)	PC 3 (5.74%)	PC 4 (3.61%)
Spur Length	**0.22071**	**0.37075**	0.047595	-0.03864
Median Sepal Length	**0.2476**	**-0.38123**	-0.10339	**0.21566**
Median Sepal Width	**0.2653**	-0.05814	0.01141	**-0.59196**
Lateral Sepal Length	**0.28891**	**-0.22474**	-0.0179	0.084359
Lateral Sepal Width	**0.26105**	0.23358	0.032796	**-0.44593**
Petal Length	**0.29349**	-0.16996	-0.07093	**0.26383**
Petal Width	**0.30243**	-0.13341	-0.03354	-0.04032
Labellum Lip Length	0.11493	**-0.2432**	**0.9227**	0.11593
Labellum Lip Width	**0.26707**	-0.09427	0.070926	**-0.33315**
Ovary Length	0.1116	**0.5407**	**0.25737**	0.171
Column Length	**0.22313**	**0.40423**	0.010243	**0.28138**
Stigma Flap Length	**0.30157**	0.070716	-0.03771	0.067523
Stigma Flap Width	**0.29741**	-0.04392	-0.05797	-0.0223
Rostellum Length	**0.29114**	0.14911	-0.10668	**0.20126**
Rostellum Width	**0.28185**	-0.09548	-0.20112	**0.21442**

Table 2.6: Mean ± SD of measured traits (mm) of the high and low-elevation forms of ssp. *neglectum* and ssp. *woodii*. Statistical significance between the means is: NS – non-significant, * P <0.05, ** P < 0.01, ***P < 0.005

Trait				**P[1]**		
	High	Low	ssp. *woodii*	H vs. L	H vs. W	L vs. W
Spur Length	9.18 ± 1.09	10.69 ± 1.34	14.94 ± 1.59	***	***	***
Median Sepal Length	6.97 ± 0.73	4.55 ± 0.6	8.68 ± 1.09	***	***	***
Median Sepal Width	2.04 ± 0.2	1.87 ± 0.28	2.65 ± 0.31	***	***	***
Lateral Sepal Length	6.61 ± 0.6	5.26 ± 0.55	8.72 ± 0.99	***	***	***
Lateral Sepal Width	2.25 ± 0.42	2.46 ± 0.3	3.94 ± 0.61	NS	***	***
Petal Length	6.37 ± 0.83	4.54 ± 0.68	9.83 ± 1.29	***	***	***
Petal Width	2.37 ± 0.27	1.65 ± 0.27	3.83 ± 0.35	***	***	***
Labellum Lip Length	2.04 ± 0.33	1.83 ± 0.3	2.14 ± 0.34	*	**	***
Labellum Lip Width	2.41 ± 0.28	2.03 ± 0.31	3.47 ± 0.83	***	***	***
Ovary Length	5.99 ± 1.08	8.66 ± 1.73	10.18 ± 2.25	***	***	***
Column Length	3.4 ± 0.57	4.75 ± 0.75	8.19 ± 1.48	***	***	***
Stigma Flap Length	1.04 ± 0.22	0.84 ± 0.21	2.39 ± 0.44	*	***	***
Stigma Flap Width	1.93 ± 0.27	1.47 ± 0.23	3.33 ± 0.55	***	***	***
Rostellum Length	1.56 ± 0.27	1.45 ± 0.25	3.71 ± 0.43	*	***	***
Rostellum Width	1.36 ± 0.16	1.07 ± 0.19	2.09 ± 0.34	**	***	***

[1] H = High-form; L = Low-form; W = ssp. *woodii*

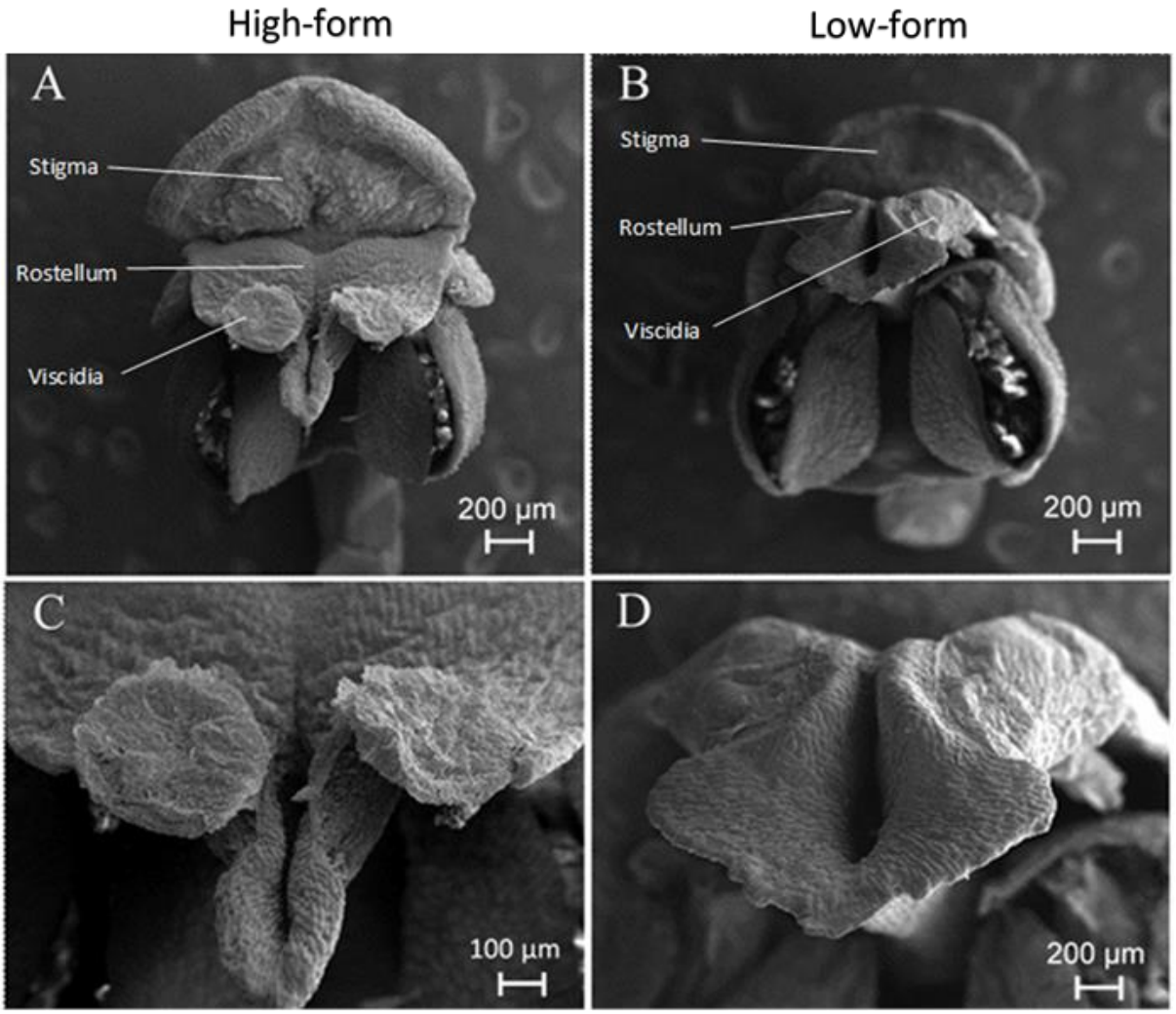

Figure 2.10: Scanning Electron Microscopy of the rostellum and stigma of the high- and low-elevation form of ssp. *neglectum* (Mt Aux-Sources: A and C; Bushmen's Nek: B and D).

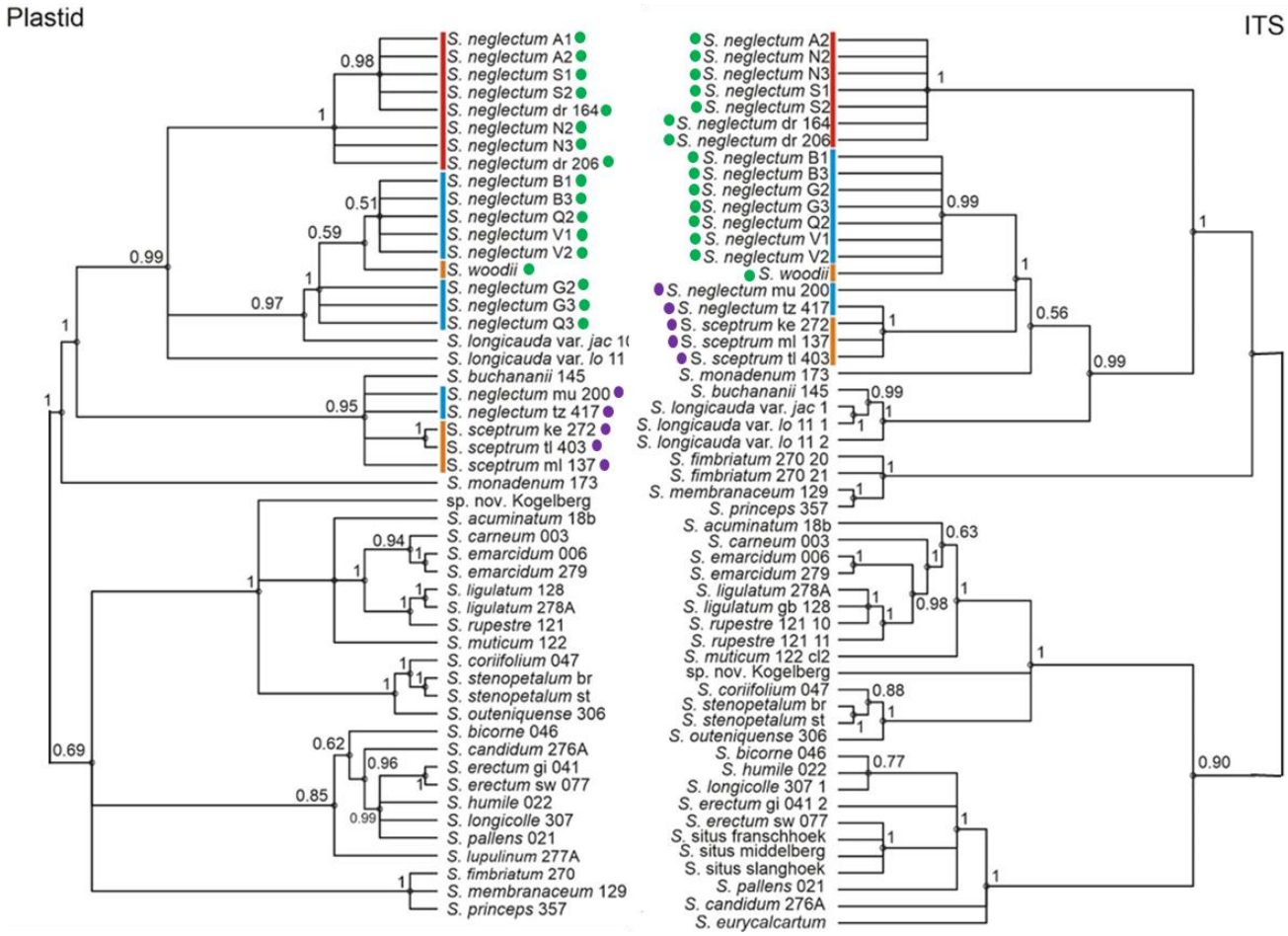

Figure 2.11: Bayesian Inference majority consensus tree of *Satyrium* analysed from plastid (trnL-F, trnSG and matK) and nuclear (Internal Transcribed Spacer) DNA sequences (Red = high-elevation form; blue = low-elevation form and orange = ssp. *woodii;* Green circles =South African accessions; Purple circles = eastern African accessions). Numbers above branches represent posterior probability.

2.5 Discussion

This study finds that the southern African high-elevation form differs in morphology from both the low-elevation form and from ssp. *woodii*. The high-elevation form also is an independent, a monophyletic clade, despite incongruencies between nuclear and plastid data sets. The cumulative evidence suggests that the high-elevation form is an independent species, separate from both the low-elevation form and from ssp. *woodii*. One of the main aims of this study was to evaluate whether the high-elevation form of ssp. *neglectum* in South Africa constitutes a different species. This question was approached by implementing a quantitative morphological analysis, similar to what was done by Hall (1982), but by specifically considering the geographical origin of the specimens, and by expanding the phylogenetic analysis done by Van der Niet and Linder (2008) with denser sampling of the high-elevation form and through the inclusion of samples from the low-elevation form from South Africa.

Results from the morphological analysis are largely consistent with those of Hall (1982). If only samples from South Africa are considered, there are clear differences between the high- and low-elevation forms (Figure 2.3 & Figure 2.8), but if samples from the northern range of ssp. *neglectum* are included, the differences seem to become less pronounced (Figure 2.2). Nevertheless, significant differences based on multivariate traits analysis appear to be present between the high-elevation form and other entities of ssp. *neglectum* (Table 2.2), and specimens of this form differ significantly in several traits (Table 2.4 & Table 2.6; Figure 2.9).Though the variation explained by the principal components axes were low in the herbarium-based analysis, this is most likely the result of measurement error, likely due to the distortion of herbarium specimens as they dry and are pressed. In addition, though no single quantitative trait unambiguously distinguishes the high-elevation form from the low-elevation form, several qualitative traits clearly set them apart and could be used as distinguishing traits in fresh and preserved specimens. In future analyses, qualitative traits could be added into a principal components analysis implementing the Gower similarity index, which may provide a clearer separation in the herbarium-based analysis. Such qualitative traits include the slight transparency of flowers and reflexion of the sepals and petals, with the free parts of flowers in the high-elevation form, which appear spreading and perpendicular to the galea aperture, as opposed to being greatly reflexed in both the low-elevation form and ssp. *woodii*. The intensity of colour varies slightly in the high-elevation form. However, the white cream-coloured flowers which are tinged with purple along the centre of the sepals and petals, are representative

and distinctive, and clearly differ from the deep pink and orange/red found in the low-elevation form and ssp. *woodii* respectively. Furthermore, the leaves are often not collected, but differ, with the low-elevation form having leaves that are generally much longer, then the rounded, generally much smaller leaves found in the high-elevation form. Based on the evidence outlined above, I therefore argue that the high-elevation form constitutes a distinct morphological entity, in contrast to Hall (1982).

A new line of evidence that further supports the distinct status of the high-elevation form, is the expanded phylogenetic analysis. Van der Niet and Linder (2008) already presented results that supported polyphyly of ssp. *neglectum*, due to the distinct phylogenetic position of accessions of the high-elevation form. However, their taxon sampling only included two accessions of the high-elevation form and did not include accessions of the low-elevation form from South Africa. All high-elevation form samples included here group together in a monophyletic clade. Furthermore, the polyphyly of ssp. *neglectum* is further reinforced in the current analysis, in which the high-elevation samples occupy a distinct phylogenetic position. If Hall's (1982) suggestion that populations from the northern part of the range form a bridge between the low- and high-elevation populations in South Africa is correct, a different phylogenetic pattern would have been expected, in which ssp. *neglectum* is monophyletic, with affinities between the high-elevation form and samples from the northern part of the range. However, this clearly is not the case. In future studies it would be useful to include samples representing var. *brevicalcar* from Malawi and Tanzania. Based on the morphological similarity between specimens of var. *brevicalcar* and ssp. *neglectum* from East Africa, I predict that they will group with these accessions. From a geographical point of view this would also be likely: if they were sister to the high-elevation form from SA, it would mean that the distribution of the two forms is characterized by a large disjunction. This would possibly represent extinction of intermediate populations along high-elevation environments such as Mt Mulanje and Nyika plateau which are currently y characterized by high orchid diversity including the low-elevation form. If specimens of var. *brevicalcar* were to group with low-elevation accessions, the distinct morphology of var. *brevicalcar* recognized by Summerhayes (1966) likely represents an independent evolution of small flowers that occur at high-elevation.

Examination of herbarium records of the var. *brevicalcar*. a synonym of ssp. *neglectum*, finds that the concept is different from the high-elevation form in several floral and vegetative traits. Overall, var. *brevicalcar*, has a taller and larger vegetative stem than the high-elevation form,

though it is similar in the number of flowers, and inflorescence size. Based on collector information and descriptions of the concept, the flowers of var. *brevicalcar* are described as occurring in shades of yellow or green, with brown streaks, rarely pink or pale pink. The description of flower colour in var. *brevicalcar* is vastly different from the cream, purple-tinged flowers of the high-elevation form, and shows some overlap with the low-elevation form. The overall similarity of var. *brevicalcar* with the low-elevation form could indicate that it should remain as a synonym of the low-elevation form. However, a possible reinstatement of this variety should not be overlooked. The concept, var. *brevicalcar* may represent a subspecies, alongside the low-elevation form, but differing from the SA high-elevation form, which exclusively occurs within eastern Africa, sharing some overlap in floral colour. However, fresh material, and closer inspection of var. *brevicalcar in-situ* is necessary to fully investigate this possibility. While almost all floral traits suggest a large similarity and indicate overlap between the low-elevation form and var. *brevicalcar*, the size of the inflorescence, number of flowers as well as the length of the spur, are all traits which appear to distinguish var. *brevicalcar* and the low-elevation form. This would then also mean that short spurs would have likely evolved in parallel in the high-elevation form and var. *brevicalcar*. Evolutionary lability in spur length is common in *Satyrium* (Johnson, 1997d; Castañeda-Zárate et al., 2021) and is therefore not unexpected. Spur length is one of the main characteristics which was used to distinguish the exclusively eastern African synonym, *S. neglectum* var. *brevicalcar*, from the long-spurred var. *neglectum* (Summerhayes, 1966; Hall, 1982). Indeed, differences in spur length may indicate pollinator- mediated reproductive isolation (Johnson and Steiner, 1997; Whittall and Hodges, 2007; Boberg et al., 2014)

Information on characters that could not be studied in a quantitative manner in herbarium specimens could shed further light on the species boundaries. Indeed, floral traits which were not measured on herbarium records, were characters related to the gynostemium, including the column, rostellum and stigma, all of which are lost in the pressing of floral material, and which could have contributed to the clearer distinction between groups that I and others (Parnell et al., 2013; Botes et al., 2020) obtained from analysis of ethanol preserved flowers. Characteristics related to the gynostemium are a suite of traits which are known to be critical in species delimitation of orchids and *Satyriinae* (Dressler, 1993; Kurzweil, 1996; Johnson, 1997c). The rostellum in the high-elevation form is distinctive, and consists of an extended pointed beak, as opposed to a highly flared, spathulate beak in the low-elevation form. However, more samples are required to determine if this is representative of the concept of

high-elevation form more generally. The rostellum also provides more explicit links between var. *brevicalcar* and the low-elevation form. While gynostemium measurements were not possible on herbarium pressed material, hand-drawn images on herbarium sheets (For example: KEW: J.S. Ball 792, Figure 2.12; R.E. Fries 1009), of the rostellum of var. *brevicalcar* clearly link it to the low-elevation form, as both have a similar rostellum structure, being slightly extended and featuring greatly flared, and spathulate rostellum beak.

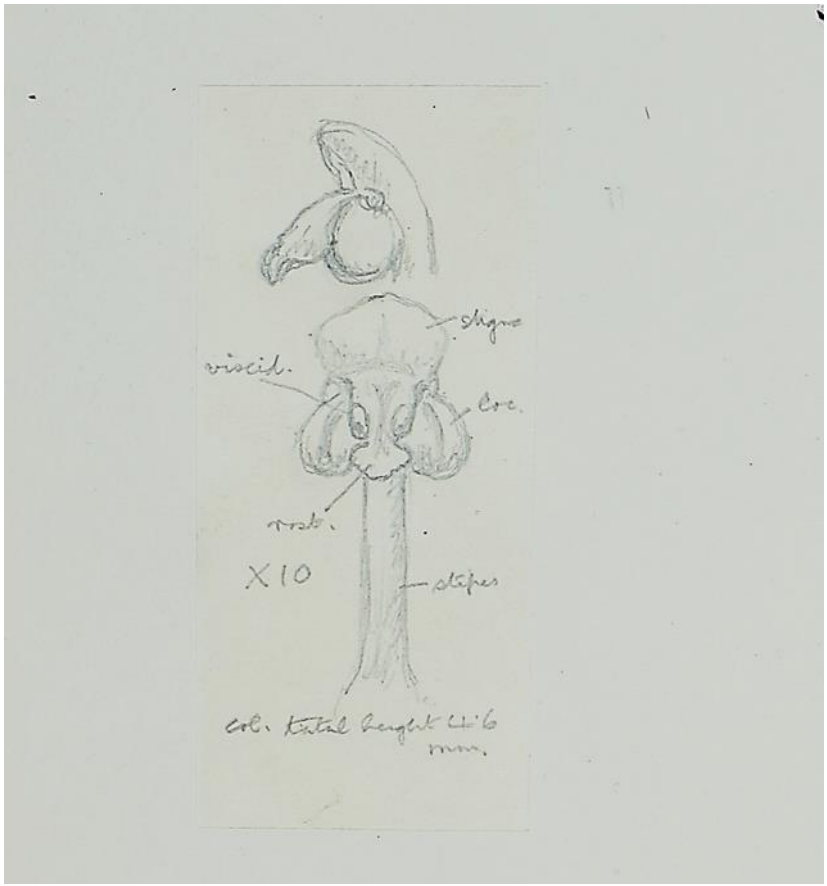

Figure 2.12. Hand-drawn image of the gynostemium structure in *Satyrium neglectum* var. *brevicalcar* from the record collected by J.S. Ball 792 (KEW).

Morphologically, the high-elevation form is easily identifiable in both herbarium and fresh material from several characteristic traits, which include the lengths of the spur, sepals and petals, as well as the much smaller inflorescence and vegetative stem. Flowers of the high-elevation form are smaller in size, with the exception of the labellum flap, which appears to be slightly larger. Once dried, the flowers of the high-elevation form become clearly transparent, and striated, providing an additional trait in the identification of herbarium samples. In addition

to morphology, the southern African high-elevation form is also geographically isolated from both the low-elevation form and from ssp. *woodii*, occurring at different elevations. The distribution of the high- and low-elevation forms along an elevation gradient suggests that elevation may have an effect on both pollinator abundance and type (Cruden, 1972; Warren et al., 1988) as well as soil type (Goldblatt and Manning, 2006; Schnitzler et al., 2011). Adaptation to differing soil types is an important ecological factor in the speciation process, affecting vegetative traits (Carbutt and Edwards, 2001; Rajakaruna, 2004). The high-elevation form is exclusively found in the highlands of the Drakensberg mountain range, and thus only occurring on basalt soil, while the low-elevation form is never found in such conditions, more commonly found in the foothills of the Drakensberg on sandstone soil (SACS, 1980; Partridge and Maud, 1987; Carbutt, 2019). Shifts in pollinator systems have often been associated with shifts in soil types and vice versa, suggesting soil type played a large part in divergence of the high-elevation form (Patterson and Givnish, 2004; Goldblatt and Manning, 2006; Johnson, 2010).

Accessions from ssp. *neglectum* and ssp. *woodii* together do not form a monophyletic clade, nor are they reciprocally monophyletic. Accessions of the low-elevation form and ssp. *woodii* are rather separated according to their geographic location. Southern African records of the low-elevation form and ssp. *woodii* are separated from their eastern African counterparts and form a monophyletic clade in both the plastid and nuclear phylogenies. The eastern African records of ssp. *woodii* represent the former synonyms *S. sceptrum* and *S. acutirostrum*. The addition of *S. sceptrum* to the concept of ssp. *woodii* is a contested subject, with some publications still recognising the former as a separate species (Pope, 1995; Van der Niet et al., 2005; Van der Niet and Linder, 2008).

The separation in accessions of the low-elevation form and ssp. *woodii* based on geographical location, was at odds with quantitative analysis of morphology and the current and previous taxonomy (Hall 1982), which suggests that ssp. *neglectum* and ssp. *woodii* are distinct subspecies. There are two possible explanations for this result: first, the distinct morphologies representing populations from South Africa versus East Africa have evolved multiple times independently. Morphological similarity would then be the result of convergence, rather than homology, for instance due to adaptation to similar pollinators as might be the case for the bird-pollinated populations of ssp. *woodii* and *S. sceptrum* (Schemske, 1981; Thomson and Wilson, 2008; Johnson et al., 2011; Anderson et al., 2014). Alternatively, gene flow within a restricted

geographical area, or lineage sorting may have resulted in incorrect estimation of species relationships. It should be noted that the non-monophyly of the two subspecies was supported by both phylogenetic datasets, although these only represent two independent gene trees. More molecular data based on additional genomic partitions are required to distinguish between the two scenarios.

The current *Satyrium neglectum* species complex should continue to be comprised by two subspecies: *S. neglectum* ssp. *neglectum* and *S. neglectum* ssp. *woodii*. The distinction between these two subspecies requires more thorough analysis, necessitating closer examination of eastern African accessions and fresh material. The *S. neglectum* species complex should comprise the following: the low-elevation form, which represents descriptions from the original concept (Schlechter, 1895) is representative of ssp. *neglectum*. The concepts which make up ssp. *woodii* (including *S. woodii, S. acutirostrum* and *S. sceptrum)* are doubtfully distinct from one another and should remain within ssp. *neglectum*. The description of the concepts and analysis of the morphology in Hall (1982) found that the differences between *S. woodii, S. acutirostrum* and *S. sceptrum* are negligible. Similar to Hall (1982), this study found that the three concepts which make up ssp. *woodii*: *S. woodii, S. acutirostrum,* and *S. sceptrum,* cannot be considered morphologically separate from one another.

2.6 Conclusion

Discrete morphological traits in combination with distinct phylogenetic placement, all suggest that the current taxonomic delimitation of the *S. neglectum* species complex is in need of revision, and that southern African populations of a high-elevation form should be recognized as a separate taxonomic entity, and therefore described as an independent species. This has obvious repercussions for conservation, as well as the removal of the concept from ssp. *neglectum*, and the status of concepts which are closely linked to it in the original description of the species. Conservation depends on accurate taxonomy to both realise the true distribution of a species following re-examination, and to recognise concepts which were hidden in synonymy (Johnson and Linder, 1995; Botes et al., 2020). Following the recognition of the south African high-elevation form as an independent species, the putative taxon can be considered rare as it is known only from a few localities, though in relatively large numbers, along the Drakensberg Mountain Range, putting it at risk from both emerging alien invasive plants, and heavy cattle grazing (O'Connor, 2005; Carbutt, 2012). This rarity could, however,

reflect the fact that the high-elevation form is generally found in inaccessible areas and thus the apparent rarity of the concept could be better explained by simple lack of collection.

The concept of *S. neglectum* should be limited to the lowland form of *Satyrium neglectum* ssp. *neglectum* along with *S. neglectum* ssp. *woodii*, the former remaining fairly common along the foothills of the Drakensberg Mountain Range with extensions into the northern provinces, Mpumalanga and Limpopo, and northwards into eastern African countries. Records of the low-elevation form are representative of the original description of *S. neglectum* by Schlechter (1895), and as such, should remain within *S. neglectum*. The synonyms, var. *brevicalcar* and *S. sceptrum* are doubtfully distinct from low-elevation form and ssp. *woodii* respectively, and a rigorous analysis requiring fresh material, is necessary to confirm or deny the reinstatement of the concepts as independent taxa. The low-elevation form and ssp. *woodii*, despite geographic separation of accessions, should remain as subspecies within *S. neglectum.* Similar to the high-elevation form, *Satyrium neglectum* ssp. *woodii* is rare in South Africa, occurring in only a few locations in Kwa-Zulu Natal, though the taxon is widespread in eastern African countries under the former synonyms *S. sceptrum* and *S. acutirostrum*.

2.7 References

Anderson, B., Ros, P., Wiese, T., Ellis, A., 2014. Intraspecific divergence and convergence of floral tube length in specialized pollination interactions. Proceedings of the Royal Society B: Biological Sciences 281, 20141420.

Barbour, R. C., O'Reilly-Wapstra, J. M., De Little, D. W., Jordan, G. J., Steane, D. A., Humphreys, J. R., Bailey, J. K., Whitham, T. G., Potts, B. M., 2009. A geographic mosaic of genetic variation within a foundation tree species and its community-level consequences. Ecology 90, 1762-1772.

Boberg, E., Alexandersson, R., Jonsson, M., Maad, J., Agren, J., Nilsson, L. A., 2014. Pollinator shifts and the evolution of spur length in the moth-pollinated orchid *Platanthera bifolia*. Annals of Botany 113, 267-275.

Botes, C., van der Niet, T., Cowling, R. M., Johnson, S. D., 2020. Is biodiversity underestimated by classical herbarium-based taxonomy? A multi-disciplinary case study in *Satyrium* (Orchidaceae). Botanical Journal of the Linnean Society 194, 342-357.

Britton, M. N., Hedderson, T. A., Verboom, G. A., 2014. Topography as a driver of cryptic speciation in the high-elevation cape sedge *Tetraria triangularis* (Boeck.) CB Clarke (Cyperaceae: Schoeneae). Molecular Phylogenetics and Evolution 77, 96-109.

Carbutt, C., 2012. The emerging invasive alien plants of the Drakensberg Alpine Centre, southern Africa. Bothalia 42, 71-85.

Carbutt, C., 2019. The Drakensberg Mountain Centre: A necessary revision of southern Africa's high-elevation centre of plant endemism. South African Journal of Botany 124, 508-529.

Carbutt, C., Edwards, T., 2001. Cape elements on high-altitude corridors and edaphic islands: historical aspects and preliminary phytogeography. Systematics and Geography of Plants, 1033-1061.

Castañeda-Zárate, M., Johnson, S. D., van der Niet, T., 2021. Food Reward Chemistry Explains a Novel Pollinator Shift and Vestigialization of Long Floral Spurs in an Orchid. Current Biology 31, 238-246. e237.

Chase, M. W., 2005. Classification of Orchidaceae in the age of DNA data. Curtis's Botanical Magazine 22, 2-7.

Cruden, R. W., 1972. Pollinators in high-elevation ecosystems: relative effectiveness of birds and bees. Science 176, 1439-1440.

Dressler, R. L. 1993. Phylogeny and classification of the orchid family. Timber Press, Portland.

Ferguson, M., Ford-Lloyd, B., Robertson, L., Maxted, N., Newbury, H., 1998. Mapping the geographical distribution of genetic variation in the genus Lens for the enhanced conservation of plant genetic diversity. Molecular Ecology 7, 1743-1755.

Freudenstein, J. V., Rasmussen, F. N., 1999. What does morphology tell us about orchid relationships? - A cladistic analysis. American Journal of Botany 86, 225-248.

Goldblatt, P., Manning, J. C., 2006. Radiation of pollination systems in the Iridaceae of sub-Saharan Africa. Annals of Botany 97, 317-344.

Hall, A. V. 1982. A Revision of the Southern African Species of *Satyrium*. Bolus Herbarium, Rondebosch, Republic of South Africa.

Hamilton, M., 1999. Four primer pairs for the amplification of chloroplast intergenic regions with intraspecific variation. Molecular Ecology 8, 521-523.

Hammer, Ø., Harper, D. A. T., Ryan, P. D., 2001. PAST: Paleontological statistics software package for education and data analysis. Palaeontologia electronica 4, 1-9.

Hodges, S. A., Arnold, M. L., 1994. Floral and ecological isolation between *Aquilegia formosa* and *Aquilegia pubescens*. Proceedings of the National Academy of Sciences, USA 91, 2493-2496.

Huelsenbeck, J. P., Ronquist, F., 2001. MRBAYES: Bayesian inference of phylogenetic trees. Bioinformatics 17, 754-755.

Johnson, S., Linder, H., 1995. Systematics and evolution of the *Disa draconis* complex (Orchidaceae). Botanical Journal of the Linnean Society 118, 289-307.

Johnson, S. D., 1997a. Insect pollination and floral mechanisms in South African species of *Satyrium* (Orchidaceae). Plant Systematics and Evolution 204, 195-206.

Johnson, S. D., 1997b. Pollination ecotypes of *Satyrium hallackii* (Orchidaceae) in South Africa. Botanical Journal of the Linnean Society 123, 225-235.

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

Johnson, S. D., Peter, C. I., Ellis, A. G., Boberg, E., Botes, C., Van der Niet, T., 2011. Diverse pollination systems of the twin-spurred orchid genus *Satyrium* in African grasslands. Plant Systematics and Evolution 292, 95-103.

Johnson, S. D., Steiner, K. E., 1997. Long-tongued fly pollination and evolution of floral spur length in the *Disa draconis* complex (Orchidaceae). Evolution 51, 45-53.

Johnson, S. D., Van der Niet, T., 2019. Bird pollination in an African *Satyrium* (Orchidaceae) confirmed by camera traps and selective exclusion experiments. Plant Systematics and Evolution 305, 477-484.

Kocyan, A., Qiu, Y. L., Endress, P. K., Conti, E., 2004. A phylogenetic analysis of Apostasioideae (Orchidaceae) based on ITS, *trn*L-F and *mat*K sequences. Plant Systematics and Evolution 247, 203-213.

Kores, P. J., Weston, P. H., Molvray, M., Chase, M. W., 2000. Phylogenetic relationships within the Diurideae (Orchidaceae): inferences from plastid *mat*K DNA sequences. Monocots: systematics and evolution, 449-456.

Kraenzlin, F., 1898. Contributions to the Flora of Africa XV. Botansche Jahrbucher fur Systematik, Pflanzengeschichte und Pflanzengeographie 24, 508-509.

Kurzweil, H., 1996. Floral morphology and ontogeny in subtribe *Satyriinae* (Fam Orchidaceae). Flora 191, 9-28.

la Croix, I. F. 1991. Orchids of Malawi. A. A. Balkema, Rotterdam.

Moriarty, A. 1975. Wild flowers of Malawi. Purnell.

O'Connor, T., 2005. Influence of land use on plant community composition and diversity in Highland Sourveld grassland in the southern Drakensberg, South Africa. Journal of Applied Ecology 42, 975-988.

Parnell, J., Rich, T., McVeigh, A., Lim, A., Quigley, S., Morris, D., Wong, Z., 2013. The effect of preservation methods on plant morphology. Taxon 62, 1259-1265.

Partridge, T. C., Maud, R. R., 1987. Geomorphic evolution of southern Africa since the Mesozoic. South African Journal of Geology 90, 179-208.

Patterson, T. B., Givnish, T. J., 2004. Geographic cohesion, chromosomal evolution, parallel adaptive radiations, and consequent floral adaptations in *Calochortus* (Calochortaceae): evidence from a cpDNA phylogeny. New Phytologist 161, 253-264.

Pope, G. V. 1995. Flora Zambesiaca: Mozambique, Malawi, Zambia, Zimbabwe, Botswana. Volume 11: Part 1. Flora Zambesiaca Management Committee.

Posada, D., Crandall, K. A., 1998. Modeltest: testing the model of DNA substitution. Bioinformatics (Oxford, England) 14, 817-818.

Rajakaruna, N., 2004. The edaphic factor in the origin of plant species. International Geology Review 46, 471-478.

Rambaut, A., Drummond, A. J., Xie, D., Baele, G., Suchard, M. A., 2018. Posterior summarization in Bayesian phylogenetics using Tracer 1.7. Systematic Biology 67, 901.

Raven, P. H., 2004. Taxonomy: where are we now? Philosophical Transactions of the Royal Society of London. Series B: Biological Sciences 359, 729-730.

Rolfe, R. A. 1898. *Satyrium densum*. Pages 270-271.In T.-D. W.T., ed. Flora of Tropical Africa. Lovell Reeve and Co. Limited, London.

Ronquist, F., Huelsenbeck, J. P., 2003. MrBayes 3: Bayesian phylogenetic inference under mixed models. Bioinformatics 19, 1572-1574.

SACS, S., 1980. Stratigraphy of South Africa, Part I (Compiler LE Kent). Lithostratigraphy of RSA, SWA/Namibia and Rep. of Bophuthatswana, Transkei and Venda. Handbook Geological Survey of South Africa 8.

Schemske, D. W., 1981. Floral convergence and pollinator sharing in two bee-pollinated tropical herbs. Ecology 62, 946-954.

Schlechter, R., 1895. Contributions to the knowledge of new and critical orchids from South Africa. Botansche Jahrbucher fur Systematik, Pflanzengeschichte und Pflanzengeographie 20.

Schlechter, R., 1915. Contributions to the Flora of Africa XLV. Botansche Jahrbucher fur Systematik, Pflanzengeschichte und Pflanzengeographie 53, 527-529.

Schlechter, R. 1916. *Satyrium papyretorum*. Pages 243-244 Der Schwedischen Rhodesia-Kongo Expedition. Aftonbladets Druckerei, Stockholm.

Schnitzler, J., Barraclough, T. G., Boatwright, J. S., Goldblatt, P., Manning, J. C., Powell, M. P., Rebelo, T., Savolainen, V., 2011. Causes of Plant Diversification in the Cape Biodiversity Hotspot of South Africa. Systematic Biology 60, 343-357.

Sosef, M. S. M., Degreef, J., Engledow, H., Meerts, P. 2020. Botanical classification and nomenclature - an introduction. Meise Botanic Garden, Belgium.

Summerhayes, V., 1966. African orchids: XXX. Kew Bulletin 20, 165-199.

Summerhayes, V. S., 1931. African orchids, II. Bulletin of Miscellaneous Information (Royal Botanic Gardens, Kew) 1931, 378-390.

Taberlet, P., Gielly, L., Pautou, G., Bouvet, J., 1991. Universal primers for amplification of three non-coding regions of chloroplast DNA. Plant Molecular Biology 17, 1105-1109.

Thomson, J., Wilson, P., 2008. Explaining evolutionary shifts between bee and hummingbird pollination: convergence, divergence and directionality. International Journal of Plant Sciences.

Van der Niet, T., 2017. *Satyrium liltvedianum*: A newly discovered orchid species from the Kogelberg Mountains of the Cape Floristic Region (South Africa). South African Journal of Botany 111, 126-133.

Van der Niet, T., Liltved, W., Johnson, S. D., 2011. More than meets the eye: a morphological and phylogenetic comparison of long-spurred, white-flowered *Satyrium* species (Orchidaceae) in South Africa.

Van der Niet, T., Linder, H., 2008. Dealing with incongruence in the quest for the species tree: a case study from the orchid genus *Satyrium*. Molecular Phylogenetics and Evolution 47, 154-174.

Van der Niet, T., Linder, H., Bytebier, B., Bellstedt, D. U., 2005. Molecular Markers Reject Monophyly of the Subgenera of *Satyrium* (Orchidaceae). Systematic Biology 30, 263-274.

Warren, S. D., Harper, K. T., Booth, G. M., 1988. Elevational Distribution of Insect Pollinators. American Midland Naturalist 120, 325-330.

White, T. J., Bruns, T., Lee, S., Taylor, J., 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. PCR protocols: a guide to methods and applications 18, 315-322.

Whittall, J. B., Hodges, S. A., 2007. Pollinator shifts drive increasingly long nectar spurs in columbine flowers. Nature 447, 706-U712.

CHAPTER 3 : COMPARATIVE ANALYSIS OF FLORAL SCENT CHEMISTRY AND POLLINATION ECOLOGY IN THE *SATYRIUM NEGLECTUM* SPECIES COMPLEX

3.1 Abstract

Forms of species complexes which are difficult to distinguish based on morphology may have diverged in traits which are typically not used in taxonomy. In plants, these may include floral traits important for pollination such as scent chemistry. Floral scent was investigated in combination with published and novel pollinator observations in the *Satyrium neglectum* species complex. Comparative multivariate analysis of floral headspace scent, taken from published data for the bird-pollinated *S. neglectum* ssp. *woodii* and sampled in the field for the high- and butterfly-pollinated low-elevation forms of ssp. *neglectum*, revealed a complete separation in scent composition. Of the total 76 compounds found in the scent profiles of both forms, and ssp. *woodii*, 14 were unique to the high-elevation form, 22 were unique to the low-elevation form, and 12 were unique to ssp. woodii. Aliphatic alcohols were the dominant compound class in both forms, followed by benzenoids & phenylpropanoids and aliphatic esters in the high and low-elevation form respectively. Emission rates varied within the species complex: Mean total emission (μgh-1) being the highest in the low-elevation form, followed by the high-elevation form, and then within ssp. *woodii*. However, day-time emission rates did not vary between the high-elevation form and ssp. *woodii*. The scent profile of the high-elevation form was dominated by phenylethyl alcohol and eugenol, whereas the blend of the low-elevation form was dominated by hexan-1-ol, octan-1-ol and benzyl alcohol. In addition to previously published observations of long-proboscid fly pollination in the high-elevation form, four settling moths were observed on two different occasions (9th and 15th February 2020) visiting flowers in a population at Naude's Nek. Electroantennographic responses in two of these to phenylethyl alcohol and eugenol indicate that floral scent could be functional for adaptation for moth-pollination, in combination with the relatively large amount of scent per flower, and given that plant species that are specialized for long-proboscid fly pollination are usually unscented. Members of the *Satyrium neglectum* species complex from South Africa are characterized by different pollination systems, associated with distinct floral scent profiles. The divergence in floral traits and pollination systems further reinforces the recognition of the high elevation form as a separate species. Future work should focus on pollination ecology and floral

traits of populations in other parts of the range where taxonomic boundaries are particularly unclear.

Keywords: *Pollination; Satyrium; moth pollination; settling; long-proboscid fly, EAD; spur length*

3.2 Introduction

Convergent evolution in floral traits is often explained by the selective pressure promoted by the mostly mutualistic interaction between plants and their pollinator(s) (Grant, 1949; Galen, 1999; Johnson and Steiner, 2000; Fenster et al., 2004). The dependence on pollinators for the transfer of pollen consequently allows for pollinator-mediated trait selection to shape floral morphology, colour, scent composition and floral reward (Waser and Campbell, 2004; Mant et al., 2005; Johnson, 2006; Schiestl and Schluter, 2009). Functional traits are defined as characteristics which can impact the three fundamental cornerstones of evolutionary success: growth, reproduction and survival (Violle et al., 2007), and may encompass either physiological, morphological and phenological characters (Faegri and van der Pijl, 1979; Violle et al., 2007). In flowers, functional traits can generally affect plant-pollinator interactions, and are separated into primary attractants, which are those that can be defined as a floral reward, or secondary attractants, which are involved in the initial advertisement or attraction of pollinators to the plant (Faegri and van der Pijl, 1979). The interaction between plant and pollinator can thus affect the advertisement of rewards, access mechanisms and even the rewards themselves (Grant, 1949; Galen, 1999; Johnson, 2006; Schiestl and Schluter, 2009; Armbruster et al., 2014).

Traditional taxonomy and systematics relies on the use of floral traits for species delimitation, often using morphological differences between reproductive structures as a means for evaluating taxonomic relationships (Anderson et al., 2002). Despite this, pollination ecology and breeding systems are not often used in combination with systematics and species descriptions, even though these traits are used for species delimitation (Anderson et al., 2002). For instance, in *Aquilegia* functional floral traits such as spur length and colour (Hodges and Arnold, 1995; Hodges, 1997) have been associated floral divergence. In other systems it has been shown that subtle changes in scent chemistry can lead to major changes between pollination systems, often resulting in reproductive isolation, and speciation (Van der Niet et al., 2014; Castañeda-Zárate et al., 2021). Classical herbarium taxonomy is limited to traits

visible in herbarium records, and subject to loss of important diagnostic traits, which have not or cannot be included in herbarium sheets (Gentry, 1989). The estimation of species boundaries may become more accurate upon the addition of important traits associated with pollination, such as scent composition.

Scent plays an integral part in numerous pollination systems (Proctor et al., 1996; Andersson, 2001; Knudsen et al., 2006; Johnson and Jürgens, 2010; Van der Niet et al., 2014), and functions in combination with colour and shape in long-distance attraction, in addition to signalling the presence of a reward, or even directing pollinators to specific parts of a flower (Dobson et al., 1999; Dobson and Bergström, 2000; Knudsen, 2002; Raguso, 2004). Indeed, it has been shown that scent alone is capable of attracting pollinators without the presence of floral structures (Pellmyr, 1986), and is often central in specialised pollination systems (Knudsen and Tollsten, 1995; Dobson, 2006; Johnson and Jurgens, 2010). The association of certain scent compounds with particular pollinator groups has been studied extensively, illustrating its importance in numerous plant-pollinator interactions (Knudsen and Tollsten, 1995; Dobson, 2006; Johnson and Jurgens, 2010).

The species complex *Satyrium neglectum* is considered to be an extremely variable group and is currently comprised of two subspecies: ssp. *neglectum* and ssp. *woodii* (Hall, 1982). Hall's (1982) revision of the genus, resulted in the combination of var. *neglectum* (Summerhayes, 1966), with a short-spurred Tanzanian variety, var. *brevicalcar*, which is also represented in the high-lands of Lesotho and South Africa, into the current ssp. *neglectum* (Summerhayes, 1966; Hall, 1982). Additionally, the concepts, *S. woodii* (Schlechter, 1895), *S. sceptrum* (Schlechter, 1915), and *S. acutirostrum* (Summerhayes, 1931), were all relegated to synonymy under ssp. *woodii* (Hall, 1982). Some authors however still consider *S. sceptrum* to be separate from ssp. *woodii*, being readily identified in the field (Pope, 1995; Van der Niet et al., 2005; Van der Niet and Linder, 2008; Johnson et al., 2011). An expanded phylogenetic and morphological analysis suggests that the short-spurred, southern African high-elevation form is different from both the low-elevation form of ssp. *neglectum* and from the eastern African var. *brevicalcar*. However, for simplicity's sake, the southern African high- and low-elevation forms will still be referred to as such (Chapter 2).

The pollination system in the *S. neglectum* species complex has been partially studied. For instance, bird pollination in ssp. *woodii* has been firmly established in Johnson and Van der Niet (2019), through a combination of Camera-trap footage which demonstrated pollinarium deposition on the bills of both the amethyst and the greater-double-coloured sunbirds, and

through pollinator exclusion experiments which indicated lower pollinarium removal in individuals where birds were excluded. Furthermore, *S. sceptrum*, was also observed being visited by sunbirds (Johnson et al., 2011). The relatively large, robust inflorescence with orange-red, weakly scented flowers of ssp. *woodii* are consistent with bird pollination syndrome (Faegri and van der Pijl, 1979; Dobson, 2006). Observations of the brightly coloured flowers of the low-elevation form found that it was visited by two species of butterfly: *Acraea horta* (Nymphalidae) and *Colias electo* (Pieridae), both of which were observed carrying pollinaria (Johnson et al., 2011). Scent has been shown to play an integral part, in the interaction, with evidence suggesting that butterflies rely on floral scent for foraging and are able to associate both natural and synthetic floral scent compounds with rewards (Andersson et al., 2002; Andersson, 2003; Andersson and Dobson, 2003; Dobson, 2006), though to the human-nose, the low-elevation form does not possess any discernible scent.

Observations of the high-elevation form at a population in Mont. Aux-Sources found visitation by a long-tongued fly: *Prosoeca ganglbaueri* (Nemestrinidae) (Johnson et al., 2011). Fly pollination at high elevations has been shown to be generally common along the upper levels of elevation gradients, and often represents turnover between flowers dominated by bee pollination and those pollinated by flies (Kingston and Mc Quillan, 2000; Arnold et al., 2009). Long-tongued fly pollination has been documented in numerous southern African genera, including some orchid genera, such as *Brownleea* and *Disa* (Johnson and Steiner, 1995; Manning and Goldblatt, 1996; Johnson and Steiner, 1997; Goldblatt and Manning, 2000; Johnson et al., 2011). Flowers in South Africa that have been classified under the long-tonged fly pollination syndrome are usually long-tubed, generally unscented, and usually occur in mostly pink or cream coloured flowers, but are sometimes red, pink or blue-violet in colour (Goldblatt and Manning, 2000; Woodcock et al., 2014). Contrary to the traits associated with long-proboscid fly pollination, the high-elevation form has a distinctly sweet scent and in combination with the pale white flowers and strong morphological mismatch between the fly proboscis and the much shorter spurs of the high elevation form, this suggests that the high elevation form may not be specialization for pollination by long-proboscid flies While scent is generally inconsequential in long-tongued fly pollination, it is nevertheless an extremely important factor in the interaction between certain pollinators and their flowers (Dobson et al., 1999; Knudsen, 2002; Plepys et al., 2002). The strongly and sweetly-scented flowers of the high-elevation form therefore conform to traits which are typically associated with moth pollination.

Differences in apparent pollinator groups between the high-, low-elevation form and ssp. *woodii* require an examination of functional traits which are deemed important in pollination. This chapter aims to characterise and compare important floral traits which dictate the interaction between the observed pollinators in the system. This will include a comparative analysis of the scent composition and pollinator observations.

3.3 Materials and methods

3.3.1 Pollinator Observations

Observations of the high and low-elevation forms were conducted at 3 localities in South Africa between Feb-Mar 2019 and 2020. The low-elevation form was observed at two localities: Garden Castle (29°44'41.3"S 29°12'30.4"E, ≥ 1850 m.a.s.l) and Graskop (24°56'05.7"S 30°48'22.6"E; ≥ 1600 m.a.s.l). The population at Garden Castle was relatively large with approximately 11-15 individuals spread out along the river. The population at Graskop was exceptionally small, comprised of only 4 individuals. The high-elevation form was observed at one locality: Naudes Nek (30°43'52.6"S 28°08'18.9"E, 2578 m.a.s.l). The Naudes Nek population comprises approximately ≥ 40 individuals spread out along a grassy hill at the top of the pass. The initial population found at the top of Sani Pass ((29°35'09.0"S 29°17'17.6"E; 2874 m.a.s.l) had been severely reduced in number, to the extent that not a single individual could be found when this site was revisited in 2020.

3.3.2 Floral Scent Characterisation

3.3.2.1 Scent Sampling

Floral headspace scent of the high and low-elevation form was collected between 2018 and 2020. Scent sampling occurred either *in situ* or from cut inflorescences harvested from plants from seven study sites: the low elevation form was sampled from populations from Graskop, Van Reenen's Pass (28°23'08.0"S 29°23'55.0"E), Sani Pass base (29°36'11.4"S 29°20'20.4"E, 2025 metres), Bushman's Nek (29°50'42.9"S 29°11'59.9"E), and Qacha's Nek (30°08'00.9"S 28°40'48.3"E) while the high-elevation form was sampled from Mt. Aux-Sources (28°44'56.0"S 28°53'05.0"E), Sani Pass summit (29°35'09.0"S 29°17'17.6"E 2874 m.a.s.l) and Naude's Nek. In addition, previously published scent profiles from ssp. *woodii* were included in the analysis (Johnson and Van der Niet, 2019; Online Supplementary Material 2).

Most scent sampling occurred during the day; however, to quantify potential differences in the timing of scent emissions (Theis et al., 2007; Fenske and Imaizumi, 2016), night-time scent was also sampled from both the high and low-elevation forms in some populations (Supplementary Table S5). Scent was sampled using dynamic head space extraction and were analysed using gas-chromatography-mass spectrometry (GC-MS). Prior to sampling, inflorescences which were in full flower were enclosed in a polyacetate bag (Kalle Bratschlauch Wiesbaden, Germany). Volatiles were sampled using a Personal Air Sampler for a duration of 20-24 minutes at a flow rate of 50 ml/min^{-1}. Volatiles were passed through a scent trap containing 1 mg of Tenax ® and Carbotrap ® (Sigma-Aldrich, Germany). To control for ambient air contaminants, a control was sampled at each site, from an empty polycerate bag under the same conditions and sampling time as were used for floral headspace. Samples were stored at -18°C until analysed.

3.3.2.2 Gas Chromatography-mass spectrometry (GC-MS) Analysis

Gas-chromatography of scent samples was carried out using a SCION Mass-Spectrometer (Varian, Palo Alto, California). The GC-MS was fitted with a thermal desorption device, in which the cartridges were inserted (Amirav and Dagan, 1997; Dötterl et al., 2005). The temperature program was set such that the injector began at a temperature of 40°C for three minutes, to allow for thermal desorption, and then steadily raised to 200°C as the elute passed through a Carbowax column. The GC-MS oven temperature was then raised to 240°C for 12 minutes. Compounds passed through a mass spectrometer which used electron-impact ionisation to fragment compounds and allow for identification from mass spectra fragments. Compounds were identified using the Varian Workstation Software (Version 8) and with the NIST11 Mass Spectral Library and included a comparison of Kovats Retention Index (based on comparison with compound retention times to those of a known set of alkanes), with both retention time, mass spectra and published Kovats indices. Compounds in scent samples which were also present in the control were considered air contaminants and excluded from all analyses. Relative amounts and absolute emission rates were calculated from the injection of known amounts and concentrations of three of the largest identified peaks: octan-1-ol, phenethyl alcohol, and eugenol. Two micrograms of each of these compounds were injected into a thermodesorption cartridge, which was then run in the GC-MS under the same conditions and run parameters as the scent sampled from the inflorescences. This procedure was repeated

two times. For each run, the peak area representing 2 μg of standard was calculated by determining the peak under each standard.

3.3.2.3 Statistical analysis of floral scent

To analyse and visualise variation in scent profiles between the high- and low-elevation forms of ssp. *neglectum* as well as ssp. *woodii*, a multivariate non-metric multidimensional scaling (NMDS) was used. The Bray-Curtis similarity index was used to visualise the variation between within the species complex. This index is particularly useful for data sets that contain a relatively large number of absences (Bray and Curtis, 1957). Prior to statistical analysis, data was pre-processed by determining the relative abundance of each compound from the surface area under the peaks. To compensate for the effect of dominant compounds in the analyses, proportions were square-root transformed.

A one-way ANOSIM with 9999 permutations was used to analyse differences within the species complex, comparing the high-, low-elevation form and ssp. *woodii*, in addition to a SIMPER analysis with 9999 permutations which was used to assess which volatiles contributed more than 1% Bray-Curtis similarity within the species complex. In addition, a one-way ANOSIM was be used to assess differences in scent profiles between significantly different day- and night samples in the Naude's Nek population of the high-elevation form, in combination with a SIMPER analysis. Emission rates (μgh-1) and differences in scent emissions were analysed in day-scent samples between the high-, low-elevation form and ssp. *woodii*, using a Generalised Linear Model with a Gaussian distribution and identity link function. Form was nested in site to compensate for non-independence in samples collected from the same populations, in addition to compensating for multiple comparisons with sequential Šidák correction. To determine differences between day-time emission of compounds the number of compounds in day-samples were tested with a Generalised Linear Model with a Poisson distribution and log function, with sequential Šidák correction. This analysis can be used in combination with differences in emission rates to compare scent profiles of the elevation forms with ssp. *woodii*, which is bird-pollinated, and associated with relatively fewer scent compounds, All multivariate statistical analyses were done in the program PAST v4.03 (Hammer et al., 2001) and Generalised Linear Models in SPSS v.27 (IBM Corps.).

3.3.3.4 Electroantennographic Detection

To identify volatiles in the floral scent of the high-elevation form that elicit a response in visitors caught at Naude's Nek, Gas chromatography mass spectrometry in combination with electroantennographic detection (EAD) was used (Shuttleworth et al., 2017; Johnson et al., 2020; Shuttleworth and Johnson, 2020). A Varian CP-3800 gas chromatograph fitted with a flame-ionization detector coupled together with an EAD (Ockenfeks Syntech, Germany). Antennae were mounted in-between two glass pipettes filled with quarter-strength Ringer solution. The GC was fitted with two non-polar capillary columns which were connected to a splitter allowing the effluent to pass over both the antennae and through the GC-MS-FID machine for identification (Shuttleworth and Johnson, 2020). Major compounds which elicited a response in the EAD were identified by comparison of retention times with those of synthetic standards injected on the GC-FID system. Two EAD runs were conducted in total on the two settling moths which were caught in the evening and stored in the fridge overnight prior to their use.

3.4 Results

3.4.1 Pollination observations

Night-time observations of the high-elevation form at Naude's Nek revealed four records of visitation of settling moths on two different evenings (9th and 15th February at approximately 19.00), of which two settling moths were caught (Table 3.1). No pollinaria were observed on proboscides of the moths visiting, or on the two that were caught. Day-time observations in the same population did not reveal any visitors. Pollinarium removal experiments were started, but owing to weather conditions making the population at Naude's Nek inaccessible, could not be completed, however no removal was observed in flowers marked for the time the experiment was run. Day- and night-time observations of populations of the low-form at both Garden Castle, and Graskop respectively, did not reveal any visits. Butterflies were found occurring in large numbers in the population at Garden's Castle, but were only seen visiting nearby *Scabiosa* sp. flowers.

3.4.2 Floral Scent Characterisation

The scent bouquet of the high-elevation form consisted of 33 compounds, whilst the low-elevation form comprised of 55 compounds (Table S5). Of the compounds identified, the vast majority were aliphatic alcohols for both forms, while the second most frequently measured compounds were benzenoids and phenylpropanoids in the high-elevation form, and aliphatic esters in the low-elevation form.

Major scent volatiles in the scent bouquet of the high-elevation form are phenylethyl alcohol (90.57% ± 3.03), eugenol (6.1% ± 3.91), phenylacetaldehyde (0.41% ± 0.16), and 2-phenylethyl formate (0.2% ± 0.08) (Table 3.2; Table S5). Of the scent compounds detected in the low-elevation form, the four highest were: hexan-1-ol (71.84% ± 20.08), octan-1-ol (4.36% ± 4.05), n-butanol (3.03% ± 3.69) and benzyl alcohol (9.77% ± 18.73) (Table 3.2; Table S4). Dominant compounds in the scent bouquet of ssp. *woodii* included: 3-hexen-1-ol (16.9% ± 5), heptanal (12.6% ± 1.41), hexan-1-ol (11.63% ± 5.52) and linalool (12.03% ± 7.46). When the scent profile is plotted in 2-dimensional space, the difference between scent profiles is apparent, and it is clear that there is no overlap (Figure 3.1). Scent composition between the two forms is significantly different (ANOSIM: p-value = 0.0001; R = 0.9998), as is the scent composition of the Low-form versus ssp. *woodii* (ANOSIM: p-value = 0.0005; R = 0.9836), and of the high-form versus ssp. *woodii* (ANOSIM: p-value = 0.0014; R = 1). Scent composition of night and day samples of the high-elevation form at Naude's Nek did not differ significantly (ANOSIM: p-value = 0.1445; R = 0.1647; permutations = 9999).

Comparison of scent compounds contributing to differences based on Bray-Curtis dissimilarity found overall dissimilarity of 93.24%. The two compounds which contributed the most to the dissimilarity between the high-and low-elevation forms were phenylethyl alcohol and hexan-1-ol, respectively, followed by octan-1-ol and eugenol (Table 3.2). SIMPER Bray-Curtis dissimilarity between the low-elevation form and ssp. *woodii* found overall dissimilarity of 77.64%. The main compounds which contributed to differences between the low-form and ssp. *woodii* were heptanal, 3-hexen-1-ol, and hexan-1-ol, (Table 3.3). Overall SIMPER Bray-Curtis dissimilarity between the high-elevation form and ssp. *woodii* was 89.26%. The largest contributing compounds to this difference were: phenylethyl alcohol and 3-hexen-1-ol (Table 3.4)

The largest average scent emission calculated from the inflorescences was measured from a population of the low-elevation form from Qachas's Nek (37.25 ± 49.51 μg/h^{-1}), followed by the second highest measured emission rate from the population of the high-elevation form at Naude's Nek (0.43 ± 0.47 μg/h^{-1}), while the lowest emission was calculated from ssp. *woodii* (0.0378 ± 0.0043 μg/h^{-1}). A generalised linear model comparing the total emission rates (μgh-1) of day-time samples calculated from whole inflorescences, indicated significant difference between the high- and low-elevation form ($p < 0.0005$), and between the low-elevation form and ssp. *woodii* ($p = 0.001$), but non-significant differences between the high-elevation form and ssp. *woodii* ($p = 0.526$). A Generalised Linear Model used for comparing the total number of compounds of day-time samples, indicated a significant difference between the high-elevation form and both the low-elevation form ($p = 0.018$), and ssp. *woodii* ($p = 0.018$). The number of compounds between the low-elevation form and ssp. *woodii* did not differ ($p = 0.466$).

Electroantennographic detection using two settling moths, caught visiting the high-elevation form, showed several responses to compounds in the scent profile of the high-elevation form. Both specimens of moth elicited responses to phenylethyl alcohol and eugenol (Figure 3.2).

Table 3.1. Pollinator Proboscis length (mm)ff floral visitors captured in populations of the *Satyrium neglectum* species complex and orchid spur length (mm) measured in previous studies and this one

Locality	Spur Length ($\bar{X}$ ± SD)	Pollinator/Visitor	Proboscis Length ($\bar{X}$ ± SD)	Source
High-form				
Mt. Aux-Sources	8.89 ± 1.16	*Prosoeca ganglbaueri*	19.8 ± 2.4	Johnson et al. (2011)
Naude's Nek	9.4 ± 1.08	Moth sp. 1 (large)	9.2	This study
		Moth sp. 2 (small)	6.69	This study
Sani Pass (Summit)	8.89 ± 0.98			
Low- form				
Bushman's Nek	10.23 ± 1.14			
Garden's Castle	10.23 ± 1.14			
Sani Pass (Base)	16.4 ± 3.3	*Acraea horta*	9.30 ± 1.06	Johnson et al. (2011)
		Colias electo	9.97 ± 1.14	
Van Reenen's Pass	12.37 ± 1.4			
ssp. *woodii*				
		Amethyst Sunbird	25–35 mm	Downs (2004); Bowie et al. (2016); Johnson and Van der Niet (2019)
Eston	14.94 ± 1.59	Greater Double-Collared Sunbird	15.8 – 19 mm	

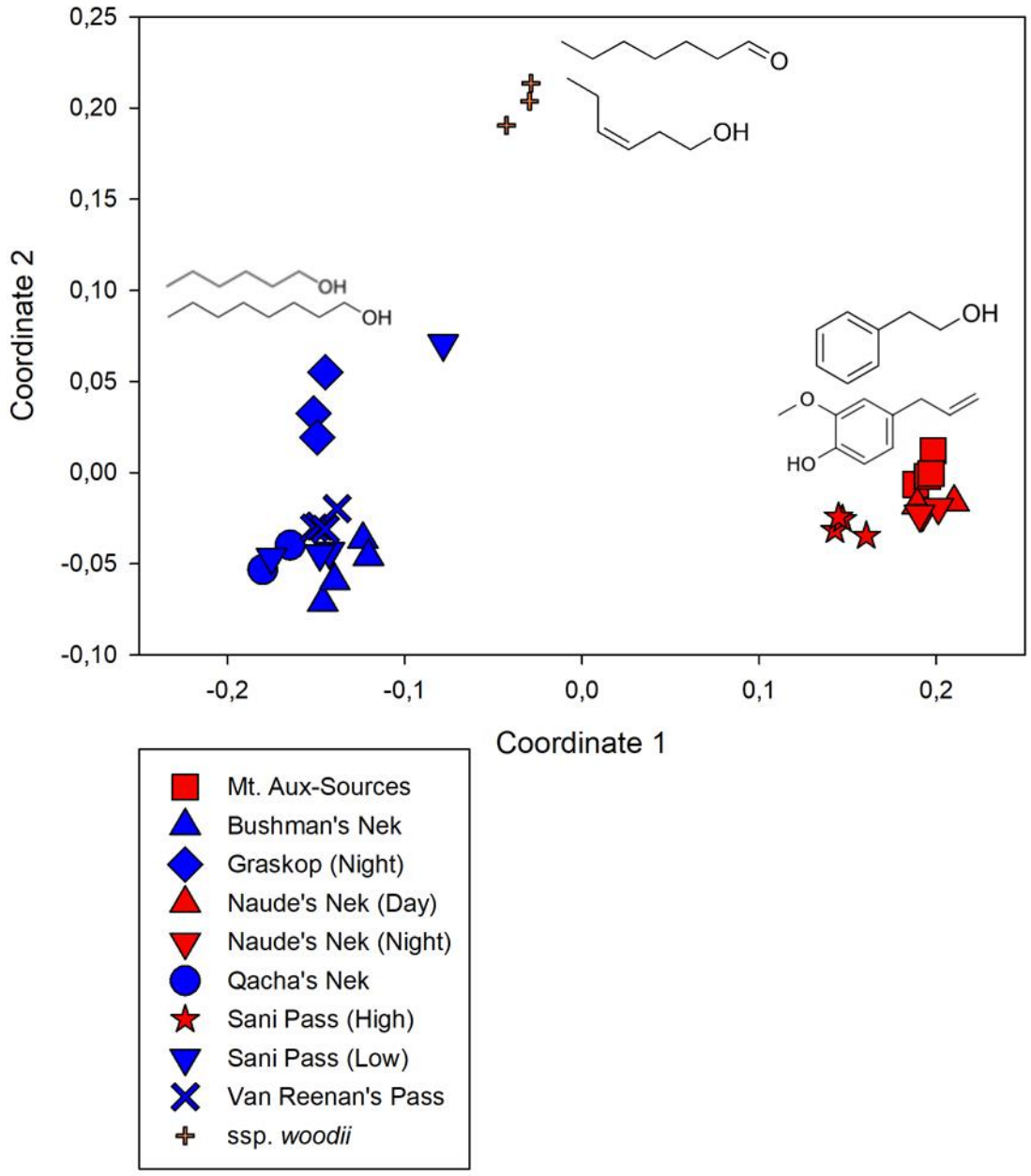

Figure 3.1: Non-metric multidimensional scaling (NMDS) ordination of floral scent profiles of *S. neglectum* ssp. *neglectum* and *S. neglectum* ssp. *woodii* (Red = High-elevation form; Blue = Low-elevation; Orange = ssp. *woodii*).

Table 3.2 Compounds which contribute ≤ 80% of average Bray-Curtis similarity between scent samples collected from *S. neglectum* ssp. *neglectum* at low (n = 17) and high-elevation (n = 14) populations (Mean represents the average relative proportion of compounds)

Taxon	Average Dissimilarity	Contribution (%)	Cumulative (%)	Mean Low-Elevation	Mean High-Elevation
Phenylethyl alcohol	25.57	27.42	27.42	0.052	0.953
Hexan-1-ol	24.3	26.06	53.48	0.862	0.016
Eugenol	5.805	6.226	59.7	0	0.204
Octan-1- ol	3.753	4.025	63.73	0.153	0.0105
Benzyl Alcohol	3.644	3.908	67.64	0.149	0.0257
n-Butanol	3.333	3.574	71.21	0.121	0
Hexyl Acetate	3.135	3.362	74.57	0.12	0
1-Butanol, 2-methyl-	2.336	2.505	77.08	0.0863	0
3-Hexen-1-ol	1.594	1.71	78.79	0.0559	0.0133
n-Heptan-1-ol	1.419	1.522	80.31	0.0612	0.00991
Phenylacetaldehyde	1.41	1.512	81.82	0	0.0511

Table 3.3: Compounds which contribute ≤ 80% of average Bray-Curtis similarity between scent samples collected from the low-elevation form (n = 17) of *S. neglectum* ssp. *neglectum* and *S. neglectum* ssp. *woodii* (n = 3) (Mean represents the average relative proportion of compounds)

Taxon	**Average Dissimilarity**	**Contribution (%)**	**Cumulative (%)**	**Mean Low-Elevation**	**Mean ssp. *woodii***
3-Hexen-1-ol	6.896	8.839	8.839	0.0557	0.515
Heptanal	6.538	8.38	17.22	0	0.449
Hexan-1-ol	6.521	8.357	25.58	0.859	0.423
Linalool	6.032	7.731	33.31	0.00439	0.42
Styrene	5.187	6.647	39.95	0	0.357
p-Cresol	4.995	6.401	46.36	0.0196	0.364
m/z: 57. 81. 67. 68. 110. 69	3.614	4.632	50.99	0	0.249
Benzyl Alcohol	3.371	4.321	55.31	0.148	0.255
Phenylethyl alcohol	3.041	3.897	59.2	0.0519	0.249
2-Octenal. (E)-	2.756	3.532	62.74	0	0.189
m/z: 79. 69. 68. 93. 67. 81	2.696	3.455	66.19	0	0.187
Octan-1-ol	2.685	3.441	69.63	0.153	0.301
2-Pentylfuran	2.469	3.164	72.8	0	0.17
m/z: 85. 81. 82. 68. 95. 67	2.065	2.647	75.44	0	0.143
m/z: 83. 68. 69. 70. 84. 67	1.965	2.518	77.96	0	0.134
m/z: 121. 81. 123. 82. 83. 93	1.923	2.464	80.43	0	0.134

Table 3.4: Compounds which contribute ≤ 80% of average Bray-Curtis similarity between scent samples collected from the high-elevation form (n = 14) of *S. neglectum* ssp. *neglectum* and *S. neglectum* ssp. *woodii* (n = 3) (Mean represents the average relative proportion of compounds)

Taxon	**Average Dissimilarity**	**Contribution (%)**	**Cumulative (%)**	**Mean High-Elevation**	**Mean ssp. *woodii***
Phenylethyl alcohol	10.82	12.12	12.12	0.953	0.249
3-Hexen-1-ol	7.767	8.699	20.82	0.0133	0.515
Heptanal	6.902	7.73	28.55	0	0.449
Linalool	6.432	7.203	35.76	0	0.42
Hexan-1-ol	6.32	7.078	42.83	0.016	0.423
Styrene	5.474	6.131	48.96	0	0.357
p-Cresol	4.997	5.596	54.56	0.0379	0.364
Octan-1-ol	4.468	5.004	59.56	0.0105	0.301
m/z: 57. 81. 67. 68. 110. 69	3.814	4.272	63.84	0	0.249
Benzyl Alcohol	3.542	3.966	67.8	0.0257	0.255
Eugenol	3.161	3.54	71.34	0.204	0
2-Octenal. (E)-	2.909	3.258	74.6	0	0.189
m/z: 79. 69. 68. 93. 67. 81	2.844	3.185	77.79	0	0.187
2-Pentylfuran	2.606	2.918	80.7	0	0.17

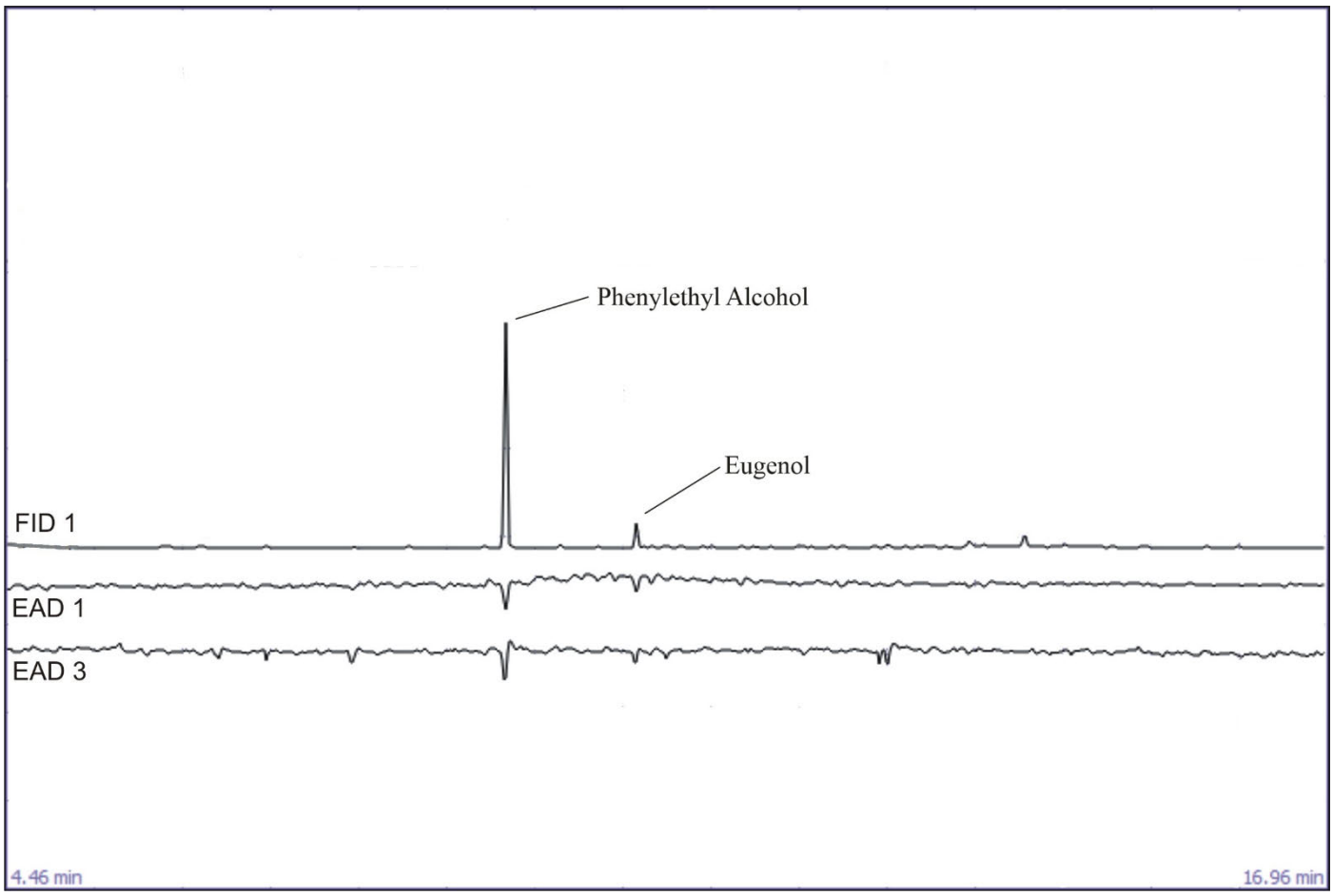

Figure 3.2: Electroantennographic Detection with antenna from two settling moths (EAD1 = Moth sp. 1; EAD3 = Moth sp. 2) using scent collected from the high-elevation form at Naude's Nek.

3.5 Discussion

This study finds that the scent composition of the high- and low-elevation forms of ssp. *neglectum* and ssp. *woodii* differ in the composition and abundance of different compound classes. Differences between dominant compound classes often arising from different biosynthetic pathways (Dudareva and Pichersky, 2006), may signal evolutionary differences between concepts. Benzenoids & Phenylpropanoids generally dominated the high-elevation form, while the low-elevation form was dominated by aliphatic alcohols, and ssp. *woodii* generally had a lack of scent, and was not dominated by any specific compound class.

In addition, this study finds that differences between floral scent compositions within the *S. neglectum* species complex is largely reflective of the apparent pollination system of the species. Pollinator-mediated trait selection is often dictated by selection which is dependent on the morphology, life-history, or sensory perception of pollinators (Harder and Johnson, 2009), and scent in pollination syndromes can result in highly specialised interactions with unique compounds attracting only specific types of pollinators (Dobson, 2006; Johnson and Jürgens, 2010; Peakall et al., 2010; Peakall and Whitehead, 2014; Gervasi et al., 2017). Indeed, in the bird-pollination syndrome, flowers are often unscented (Faegri and van der Pijl, 1979; Dobson, 2006; Johnson and Van der Niet, 2019), owing to the relatively poor olfactory perception in majority of flower visiting birds, (Dobson, 2006), with the possible exception of some hummingbirds, where learning and reward association with various compounds was demonstrated (Goldsmith and Goldsmith, 1982). Regardless, most flower-visiting birds rely on colour and shape (Hurly and Healy, 1996; Meléndez-Ackerman and Campbell, 1998). Of the concepts included, *S. neglectum* ssp. *woodii* had the least amount of floral scent compounds, conforming to bird pollination syndrome, and being confirmed in Johnson and Van der Niet (2019). Indeed, lack of scent in bird-pollination has been well documented. One such example, includes the demonstration of a shift in floral scent composition and emission rates between populations of *Erica plukenetii*, which are pollinated by moths and those pollinated by birds (Van der Niet et al., 2014), which demonstrated differences between the two associated syndromes. This is a pattern which is also represented in *Protea* where the scent emission of beetle-pollinated species was largely higher than those pollinated by birds (Steenhuisen et al., 2012).

The floral scent composition of the high-elevation form is distinct from both the low-elevation form and from ssp. *woodii*. Several compounds are unique to the scent profile of the high-elevation form, including eugenol, and trace amounts of both phenylethyl acetate and benzaldehyde, all of which are associated with scent composition of flowers pollinated by moths (Huber et al., 2005; Van der Niet et al., 2015). Prior to this study, the only visitors that were observed visiting the high-elevation form, were long-proboscis flies. Long-proboscis fly pollination was first observed on Sentinel Peak, when individuals of *Prosoeca ganglbaueri* (Nemestrinidae) were observed probing flowers of the high-elevation form (Johnson et al., 2011), and this was later supported by additional observations of individual flies visiting and carrying pollinaria reported by Johnson et al. (2011). These observations were surprising based on the mismatch between floral spur length and pollinator proboscis length, as well as the presence of scent that is more typically associated with moth pollination.

Moth pollination has previously been reported in several *Satyrium* species including: *S. acuminatum, S. ligulatum, S. outeniquense, S. pallens, S. situsanguinum* (Van der Niet et al., 2015), as well as in *S. hallackii* ssp. *hallacki* (Johnson, 1997b), *S. parviflorum* (Johnson et al., 2011), *S. stenopetalum* (Johnson, 1997a; Van der Niet et al., 2015). and *S. longicauda* (Harder and Wilson, 1998; Jersakova and Johnson, 2007; Johnson et al., 2011; Castañeda-Zárate et al., 2021). Although fragmentary, this study is the first to confirm visitation by settling moths in the high-elevation form, at a site in the southern Drakensberg. Settling moth pollination (phalaenophily) is usually associated with relatively short-spurred flowers that vary in colour, but are generally pale, and never pure white or red, with distinctly sweet floral scent (Dobson, 2006). Floral scent in settling moth-pollinated flowers functions in providing long-distance attraction, as well as direction for landing, and signalling the presence of rewards, with evidence suggesting a learning and association in moths (Cunningham et al., 2004; Raguso, 2004). The scent profile of the high-elevation form was dominated by compounds which are associated with moth pollination, including phenylethyl alcohol, eugenol, and phenylacetaldehyde (Knudsen and Tollsten, 1993; Raguso and Pichersky, 1995; Jurgens et al., 2003; Dobson, 2006). Of these compounds, both eugenol and phenylethyl alcohol had also previously been found in additional *Satyrium* species, with relatively large amounts of eugenol being reported in the moth-pollinated *Satyrium situsanguinum* (Van der Niet et al., 2009; Van der Niet et al., 2015; Johnson and Van der Niet, 2019). Furthermore, the two most dominant compounds in the scent bouquet of the high-elevation form, eugenol and phenylethyl alcohol, elicited responses in two settling moths caught on the high-elevation form. While EAD responses do not necessarily imply behavioural response, electroantennographic responses to

eugenol have also been reported in other moths and systems including, *Hyles lineata* (Sphingidae: Lepidoptera) (Raguso et al., 1996), as well as in settling moths from scent sampled from *Gymnadenia conopsea* (Orchidaceae) (Huber et al., 2005), though behavioural studies suggested no response to eugenol in the latter. Behavioural responses have been previously studied in choice experiments, producing positive responses in moths to blends containing eugenol and methyl eugenol (Gregg et al., 2010) In addition, EAD responses to phenylethyl alcohol were recorded in the moth species: *Ectropis obliqua*, though behavioural choice experiments were non-significant (Sun et al., 2014).

The presence of both moth and long-proboscid fly pollination in the high-elevation form at two disjunct populations may represent one of three scenarios. This system either represents the possibility of (1) a limitation in moth pollinator distributions, (2) opportunistic feeding by long-proboscid flies in flowers adapted for moth pollination or (3) representative of a bimodal pollination system. The co-evolution of proboscis and spur length usually occurs to promote a closer match between pollinator and flower morphology (Boberg and Agren, 2009; Boberg et al., 2014). Selection for longer spurs in flowers usually arises to increase contact with floral reproductive organs, promoting a closer mechanical fit between the spur of a flower and the corresponding proboscis length of the main pollinator (Boberg et al., 2014). Longer proboscis lengths most likely evolved to promote access to nectar, regardless of pollination efficiency in corresponding pollinators (Boberg et al., 2014). Limited distributions of main pollinators provide for the selection of traits in flowers towards available pollinators, leading to the formation of pollination ecotypes or divergence in floral characteristics (Robertson and Wyatt, 1990; Johnson, 1997b; Johnson and Steiner, 1997; Van der Niet et al., 2014). Lack of geographical variation in floral traits, such as spur length, between high-elevation populations indicates that the difference in pollinators between northern and southern populations is not a result of limited pollinator distribution range.

Associated proboscis lengths of settling moths involved in pollination in the genus *Satyrium*, ranged between 10-20 mm (Johnson et al., 2011), with the proboscis lengths of the settling moths matching the spur length in the high-elevation form at Naude's Nek, conversely, proboscis lengths of *Prosoeca ganglbaueri*, are roughly double the spur length in the high-elevation form (Johnson et al., 2011). Spur length of the high-elevation form and proboscis length of the settling moths and long-tongued flies suggests that both are able to access nectar, though mismatch between spur and proboscis lengths (Table 3.1) may suggest inefficient

pollinaria placement and deposition, as this represents physical access mechanism between pollinator and flower (Nilsson, 1988; Boberg and Ågren, 2009; Boberg et al., 2014). Identification of the pollinaria observed on individuals of *P. ganglbaueri*, were confirmed to belong to the high-elevation form, after examination of pollinarium structure in surrounding orchids (Johnson et al., 2011). Pollinaria were deposited on the lower part of the probiscis of *P. ganglbaueri*, indicating that contact is made with the rostellum and viscidia when probing (Johnson et al., 2011). However, pollination success was not examined in this population (Johnson et al., 2011), and the presence of associated moth scent compounds in the high-elevation form indicates that scent evolved to cater towards moth pollination, and long-proboscid flies may be opportunistic feeders with access to nectar in flowers which were primarily adapted for moth pollination. Opportunistic visitors have been recorded visiting flowers, which do not necessarily conform to their associated pollination syndrome (Fishbein and Venable, 1996; Miyake and Yahara, 1998), though the effectiveness of these visitors would need to be clarified to determine if they are efficient pollinators.

The observations reported in this study may also suggest the presence of a bimodal pollination system in the high-elevation form. Bimodal pollination systems are defined as those which are comprised by two different pollinator functional groups, with both groups able to contribute to successful and efficient pollination in the system (Manning and Goldblatt, 2005). Bimodal pollination in plants is not uncommon, and has been documented between a variety of pollination systems and genera, and can usually be attributed to limited distribution ranges of pollinators, leading to reliance on another pollinator group (Johnson, 1997a; Manning and Goldblatt, 2005; Monty et al., 2006; Johnson et al., 2007; Shuttleworth and Johnson, 2008; Schmid et al., 2011; Dellinger et al., 2019). The presence of both long-proboscid fly and moth visitors in the same population would confirm a true bimodal pollination system; however, pollinator observations do not provide evidence for this, as no flies were observed at the population in Naude's Nek. Given the limited observations of moth visitation in this study, this is a very cautious suggestion that requires more observations, including temporal exclusion, and examination of pollinator efficiency to conclusively substantiate.

Pollinator observations of the low-elevation form did not yield further confirmation of butterfly pollination nor any night-time visitors, though this was limited to one night. Published observations of the low-elevation form included visitation and the verification of the presence of pollinia on individuals of two butterfly species: *Acraea horta* (Nymphalidae) and *Colias*

electo (Pieridae) (Johnson et al., 2011), and butterfly pollination has been reported in a few other species in *Satyrium*, including *S. princeae* (Johnson et al., 2011) and in *S. ligulatum*, which is pollinated by butterflies and moths (Johnson, 1997a).

The largest compound in the scent profile of the low-elevation form is hexan-1-ol, which has been recorded in numerous other *Satyrium* species (Johnson et al., 2007; Van der Niet et al., 2015; Johnson and Van der Niet, 2019). Hexan-1-ol has been recorded being emitted from both leaves and bracts in other genera (Dickens, 1989; Zhang et al., 1999; Schiestl, 2015), indicating that the compound is a leafy-green volatile. However, the compound is not ubiquitous in all *Satyrium* species, indicating some functionality in pollination, as the compound has been shown to be highly attractive to some pollinator groups, such as wasps (Brodmann et al., 2012). Furthermore, both hexan-1-ol and octan-1-ol, the second largest compound in the low-elevation bouquet, are usually not released in relatively large amounts in night-time pollinated species (Dobson, 2006; Knudsen et al., 2006; Cordeiro et al., 2017), suggesting that night-time visitation in the low-elevation form is unlikely. Furthermore, the high number of aliphatic alcohols in the scent composition of the low-elevation form also conforms to the general pattern in butterfly pollination syndrome, which are generally dominated by a large number of aliphatic alcohols (Dobson, 2006).

Conversely, while benzyl alcohol, is considered an omnipresent compound, in numerous floral families (Raguso, 2004), the compound partly dominated the scent profile of the low-elevation form. The omnipresence of the compound in numerous floral genera suggested that the presence of the compound was not indicative of a particular pollination system, despite this, the compound has been found to be closely associated with butterfly pollination (Honda et al., 1998; Huber et al., 2005; Dobson, 2006). Temporal separation in scent composition is a trait which promotes attraction of certain pollinators when they are most active and circadian rhythms in floral scent emission has been demonstrated in *Satyrium* (Van der Niet et al., 2015). The pollination system in the orchid, *Gymnadenia conopsea* is comprises numerous pollinator groups, including settling moths and butterflies. A comparative examination of day- and night time samples found that while scent emission was generally lower during the night, the scent composition of *G. conopsea* differed significantly in compounds associated with moth pollination and those associated with butterfly pollination (Huber et al., 2005). Night-time scent samples had larger emission rates of compounds associated with moth pollination, including eugenol, phenylacetaldehyde and benzaldehyde (Huber et al., 2005). Day-time scent analysis

recorded an increase in the production of benzyl alcohol, when butterflies are most active and this increase is considered a trait in flowers to limit or promote visitors by controlling the timing of scent emission and production of certain compounds (Van der Niet et al., 2015; Fenske and Imaizumi, 2016).

3.6 Conclusions

This study reveals that the scent composition of the *Satyrium neglectum* complex is distinct between all taxonomic concepts, including the high-, low-elevation form and ssp. *woodii*. The scent profile of the high-elevation form is distinct from the low-elevation form, and dominated by different compound classes. Scent divergence provides further evidence for their taxonomic separation, in addition to their separation by pollination system and pollinator groups. Furthermore, this study finds that in addition to long-proboscid fly pollination, visitation by settling moths was observed in the high-elevation form. Further study in the system should revolve around confirming moths as efficient pollinators in the system and involve comparative studies between the northern and southern populations of the species, which may be representative of transition points between the pollination systems. Additional study should also focus on the understanding of the ecology of the doubtfully distinct synonyms in the *S. neglectum* species complex, including var. *brevicalcar*, which differs in both colour and possibly scent, indicating a difference in pollination system.

3.7 References

Amirav, A., Dagan, S., 1997. A direct sample introduction device for mass spectrometry studies and gas chromatography mass spectrometry analyses. European Mass Spectrometry 3, 105-111.

Anderson, G. J., Johnson, S. D., Neal, P. R., Bernardello, G., 2002. Reproductive biology and plant systematics: the growth of a symbiotic association. Taxon 51, 637-653.

Andersson, S. 2001. Floral scents in butterfly foraging and pollination. PhD thesis. Göteborg University Sweden.

Andersson, S., 2003. Antennal responses to floral scents in the butterflies *Inachis io*, *Aglais urticae* (Nymphalidae), and *Gonepteryx rhamni* (Pieridae). Chemoecology 13, 13-20.

Andersson, S., Dobson, H. E. M., 2003. Behavioral foraging responses by the butterfly *Heliconius melpomene* to *Lantana camara* floral scent. Journal of Chemical Ecology 29, 2303-2318.

Andersson, S., Nilsson, L. A., Groth, I., Bergstrom, G., 2002. Floral scents in butterfly-pollinated plants: possible convergence in chemical composition. Botanical Journal of the Linnean Society 140, 129-153.

Armbruster, W. S., Shi, X. Q., Huang, S. Q., 2014. Do specialized flowers promote reproductive isolation? Realized pollination accuracy of three sympatric *Pedicularis* species. Annals of Botany 113, 331-340.

Arnold, S. E. J., Savolainen, V., Chittka, L., 2009. Flower colours along an alpine altitude gradient, seen through the eyes of fly and bee pollinators. Arthropod-Plant Interactions 3, 27-43.

Boberg, E., Agren, J., 2009. Despite their apparent integration, spur length but not perianth size affects reproductive success in the moth-pollinated orchid *Platanthera bifolia*. Functional Ecology 23, 1022-1028.

Boberg, E., Ågren, J., 2009. Despite their apparent integration, spur length but not perianth size affects reproductive success in the moth-pollinated orchid *Platanthera bifolia*. Functional Ecology 23, 1022-1028.

Boberg, E., Alexandersson, R., Jonsson, M., Maad, J., Agren, J., Nilsson, L. A., 2014. Pollinator shifts and the evolution of spur length in the moth-pollinated orchid *Platanthera bifolia*. Annals of Botany 113, 267-275.

Bowie, R. C., Fjeldså, J., Kiure, J., Kristensen, J., 2016. A new member of the greater double-collared sunbird complex (Passeriformes: Nectariniidae) from the Eastern Arc Mountains of Africa. Zootaxa 4175, 23-42.

Bray, J., Curtis, J., 1957. An ordination of upland forest communities of southern Wisconsin. Ecological Monographs (27). Pages 325-349. PRIMER-E Plymouth.

Brodmann, J., Emer, D., Ayasse, M., 2012. Pollinator attraction of the wasp-flower *Scrophularia umbrosa* (Scrophulariaceae). Plant Biology 14, 500-505.

Castañeda-Zárate, M., Johnson, S. D., van der Niet, T., 2021. Food Reward Chemistry Explains a Novel Pollinator Shift and Vestigialization of Long Floral Spurs in an Orchid. Current Biology 31, 238-246. e237.

Cordeiro, G., Pinheiro, M., Dötterl, S., Alves-dos-Santos, I., 2017. Pollination of *Campomanesia phaea* (Myrtaceae) by night-active bees: a new nocturnal pollination system mediated by floral scent. Plant Biology 19, 132-139.

Cunningham, J. P., Moore, C. J., Zalucki, M. P., West, S. A., 2004. Learning, odour preference and flower foraging in moths. Journal of Experimental Biology 207, 87-94.

Dellinger, A. S., Scheer, L. M., Artuso, S., Fernández-Fernández, D., Sornoza, F., Penneys, D. S., Tenhaken, R., Dötterl, S., Schönenberger, J., 2019. Bimodal pollination systems in Andean Melastomataceae involving birds, bats, and rodents. The American Naturalist 194, 104-116.

Dickens, J. C., 1989. Green leaf volatiles enhance aggregation pheromone of boll weevil, *Anthonomus grandis*. Entomologia Experimentalis et Applicata 52, 191-203.

Dobson, H. 2006. Relationship between floral fragrance composition and type of pollinator. Pages 147-198.In D. N , E, P., eds. Biology of Floral Scent. CRC Press, Boca Raton: .

Dobson, H. E., Bergström, G., 2000. The ecology and evolution of pollen odors. Plant Systematics and Evolution 222, 63-87.

Dobson, H. E., Danielson, E. M., Wesep, I. D. V., 1999. Pollen odor chemicals as modulators of bumble bee foraging on *Rosa rugosa* Thunb.(Rosaceae). Plant Species Biology 14, 153-166.

Dötterl, S., Wolfe, L. M., Jürgens, A., 2005. Qualitative and quantitative analyses of flower scent in Silene latifolia. Phytochemistry 66, 203-213.

Downs, C. T., 2004. Some preliminary results of studies on the bill and tongue morphology of Gurney's Sugarbird and some southern African sunbirds. Ostrich-Journal of African Ornithology 75, 169-175.

Dudareva, N., Pichersky, E., 2006. Floral scent metabolic pathways: their regulation and evolution. Biology of floral scent 55.

Faegri, K., van der Pijl, L. 1979. The Principles of Pollination Ecology. Pergamon, Oxford.

Fenske, M. P., Imaizumi, T., 2016. Circadian rhythms in floral scent emission. Frontiers in plant science 7, 462.

Fenster, C. B., Armbruster, W. S., Wilson, P., Dudash, M. R., Thomson, J. D., 2004. Pollination syndromes and floral specialization. Annual Review of Ecology Evolution and Systematics 35, 375-403.

Fishbein, M., Venable, D. L., 1996. Diversity and temporal change in the effective pollinators of *Asclepias tuberosa*. Ecology 77, 1061-1073.

Galen, C., 1999. Why do flowers vary? The functional ecology of variation in flower size and form within natural plant populations. Bioscience 49, 631-640.

Gentry, A., 1989. Herbarium taxonomy versus field knowledge. Is there an attainable solution? Flora Malesiana Bulletin 10, 31-35.

Gervasi, D. D., Selosse, M. A., Sauve, M., Francke, W., Vereecken, N. J., Cozzolino, S., Schiestl, F. P., 2017. Floral scent and species divergence in a pair of sexually deceptive orchids. Ecology and Evolution 7, 6023-6034.

Goldblatt, P., Manning, J. C., 2000. The long-proboscid fly pollination system in southern Africa. Annals of the Missouri Botanical Garden 87, 146-170.

Goldsmith, K. M., Goldsmith, T. H., 1982. Sense of smell in the Black-chinned Hummingbird. The Condor 84, 237-238.

Grant, V., 1949. Pollination systems as isolating mechanisms in angiosperms. Evolution 3, 82-97.

Gregg, P. C., Del Socorro, A. P., Henderson, G. S., 2010. Development of a synthetic plant volatile-based attracticide for female noctuid moths. II. Bioassays of synthetic plant volatiles as attractants for the adults of the cotton bollworm, Helicoverpa armigera (Hübner)(Lepidoptera: Noctuidae). Australian Journal of Entomology 49, 21-30.

Hall, A. V. 1982. A Revision of the Southern African Species of *Satyrium*. Bolus Herbarium, Rondebosch, Republic of South Africa.

Hammer, Ø., Harper, D. A., Ryan, P. D., 2001. PAST: Paleontological statistics software package for education and data analysis. Palaeontologia electronica 4, 9.

Harder, L. D., Johnson, S. D., 2009. Darwin's beautiful contrivances: evolutionary and functional evidence for floral adaptation. New Phytologist 183, 530-545.

Harder, L. D., Wilson, W. G., 1998. A clarification of pollen discounting and its joint effects with inbreeding depression on mating system evolution. The American Naturalist 152, 684-695.

Hodges, S. A., 1997. Floral nectar spurs and diversification. International Journal of Plant Sciences 158, S81-S88.

Hodges, S. A., Arnold, M. L., 1995. Spurring plant diversification: are floral nectar spurs a key innovation? Proceedings of the Royal Society of London. Series B: Biological Sciences 262, 343-348.

Honda, K., Ômura, H., Hayashi, N., 1998. Identification of floral volatiles from *Ligustrum japonicum* that stimulate flower-visiting by Cabbage butterfly, *Pieris rapae*. Journal of Chemical Ecology 24, 2167-2180.

Huber, F. K., Kaiser, R., Sauter, W., Schiestl, F. P., 2005. Floral scent emission and pollinator attraction in two species of Gymnadenia (Orchidaceae). Oecologia 142, 564-575.

Hurly, A. T., Healy, S. D., 1996. Memory for flowers in rufous hummingbirds: location or local visual cues? Animal Behaviour 51, 1149-1157.

Jersakova, J., Johnson, S., 2007. Protandry promotes male pollination success in a moth-pollinated orchid. Functional Ecology 21, 496-504.

Johnson, S., 1997a. Insect pollination and floral mechanisms in South African species of *Satyrium* (Orchidaceae). Plant Systematics and Evolution 204, 195-206.

Johnson, S., 1997b. Pollination ecotypes of *Satyrium hallackii* (Orchidaceae) in South Africa. Botanical Journal of the Linnean Society 123, 225-235.

Johnson, S., Jürgens, A., 2010. Convergent evolution of carrion and faecal scent mimicry in fly-pollinated angiosperm flowers and a stinkhorn fungus. South African Journal of Botany 76, 796-807.

Johnson, S. D. 2006. Pollinator-driven speciation in plants. Pages 295-310.In L. D. Harder , Barrett, S. C. H., eds. The ecology and evolution of flowers. Oxford University Press, Oxford.

Johnson, S. D., Balducci, M. G., Shuttleworth, A., 2020. Hawkmoth pollination of the orchid *Habenaria clavata:* mechanical wing guides, floral scent and electroantennography. Biological Journal of the Linnean Society 129, 213-226.

Johnson, S. D., Ellis, A., Dotterl, S., 2007. Specialization for pollination by beetles and wasps: The role of lollipop hairs and fragrance in *Satyrium microrrhynchum* (Orchidaceae). American Journal of Botany 94, 47-55.

Johnson, S. D., Jurgens, A., 2010. Convergent evolution of carrion and faecal scent mimicry in fly-pollinated angiosperm flowers and a stinkhorn fungus. South African Journal of Botany 76, 796-807.

Johnson, S. D., Peter, C. I., Ellis, A. G., Boberg, E., Botes, C., Van der Niet, T., 2011. Diverse pollination systems of the twin-spurred orchid genus *Satyrium* in African grasslands. Plant Systematics and Evolution 292, 95-103.

Johnson, S. D., Steiner, K. E., 1995. Long-proboscid fly pollination of two orchids in the Cape Drakensberg mountains, South Africa. Plant Systematics and Evolution 195, 169-175.

Johnson, S. D., Steiner, K. E., 1997. Long-tongued fly pollination and evolution of floral spur length in the *Disa draconis* complex (Orchidaceae). Evolution 51, 45-53.

Johnson, S. D., Steiner, K. E., 2000. Generalization versus specialization in plant pollination systems. Trends in Ecology & Evolution 15, 190--193.

Johnson, S. D., Van der Niet, T., 2019. Bird pollination in an African *Satyrium* (Orchidaceae) confirmed by camera traps and selective exclusion experiments. Plant Systematics and Evolution 305, 477-484.

Jurgens, A., Witt, T., Gottsberger, G., 2003. Flower scent composition in *Dianthus* and *Saponaria species* (Caryophyllaceae) and its relevance for pollination biology and taxonomy. Biochemical Systematics and Ecology 31, 345-357.

Kingston, A. B., Mc Quillan, P. B., 2000. Are pollination syndromes useful predictors of floral visitors in Tasmania? Austral Ecology 25, 600-609.

Knudsen, J. T., 2002. Variation in floral scent composition within and between populations of *Geonoma macrostachys* (Arecaceae) in the western Amazon. American Journal of Botany 89, 1772-1778.

Knudsen, J. T., Eriksson, R., Gershenzon, J., Stahl, B., 2006. Diversity and distribution of floral scent. Botanical Review 72, 1-120.

Knudsen, J. T., Tollsten, L., 1993. Trends in floral scent chemistry in pollination syndromes: floral scent composition in moth-pollinated taxa. Botanical Journal of the Linnean Society 113, 263-284.

Knudsen, J. T., Tollsten, L., 1995. Floral scent in bat-pollinated plants: a case of convergent evolution. Botanical Journal of the Linnean Society 119, 45-57.

Manning, J. C., Goldblatt, P., 1996. The *Prosoeca peringueyi* (Diptera: Nemestrinidae) pollination guild in southern Africa: long-tongued flies and their tubular flowers. Annals of the Missouri Botanical Garden 83, 67-86.

Manning, J. C., Goldblatt, P., 2005. Radiation of pollination systems in the Cape genus *Tritoniopsis* (Iridaceae : Crocoideae) and the development of bimodal pollination strategies. International Journal of Plant Sciences 166, 459-474.

Mant, J., Peakall, R., Schiestl, F. P., 2005. Does selection on floral odor promote differentiation among populations and species of the sexually deceptive orchid genus *Ophrys*? Evolution 59, 1449-1463.

Meléndez-Ackerman, E. J., Campbell, D. R., 1998. Adaptive significance of flower color and inter-trait correlations in an *Ipomopsis* hybrid zone. Evolution 52, 1293-1303.

Miyake, T., Yahara, T., 1998. Why does the flower of *Lonicera japonica* open at dusk? Canadian Journal of Botany 76, 1806-1811.

Monty, A., Saad, L., Mahy, G., 2006. Bimodal pollination system in rare endemic Oncocyclus irises (Iridaceae) of Lebanon. Canadian Journal of Botany-Revue Canadienne De Botanique 84, 1327-1338.

Nilsson, L. A., 1988. The evolution of flowers with deep corolla tubes. Nature 334, 147-149.

Peakall, R., Ebert, D., Poldy, J., Barrow, R. A., Francke, W., Bower, C. C., Schiestl, F. P., 2010. Pollinator specificity, floral odour chemistry and the phylogeny of Australian sexually deceptive *Chiloglottis* orchids: implications for pollinator-driven speciation. New Phytologist 188, 437-450.

Peakall, R., Whitehead, M. R., 2014. Floral odour chemistry defines species boundaries and underpins strong reproductive isolation in sexually deceptive orchids. Annals of Botany 113, 341-355.

Pellmyr, O., 1986. Three pollination morphs in *Cimicifuga* simplex; incipient speciation due to inferiority in competition. Oecologia 68, 304-307.

Plepys, D., Ibarra, F., Francke, W., Löfstedt, C., 2002. Odour-mediated nectar foraging in the silver Y moth, *Autographa gamma* (Lepidoptera: Noctuidae): behavioural and electrophysiological responses to floral volatiles. Oikos 99, 75-82.

Pope, G. V. 1995. Flora Zambesiaca: Mozambique, Malawi, Zambia, Zimbabwe, Botswana. Volume 11: Part 1. Flora Zambesiaca Management Committee.

Proctor, M., Yeo, P., Lack, A. 1996. The Natural History of Pollination. Collins, London.

Raguso, R. A., 2004. Why do flowers smell? The chemical ecology of fragrance-driven pollination. Advances in insect chemical ecology, 151-178.

Raguso, R. A., Light, D. M., Pickersky, E., 1996. Electroantennogram responses of *Hyles lineata* (Sphingidae: Lepidoptera) to volatile compounds from *Clarkia breweri*

(Onagraceae) and other moth-pollinated flowers. Journal of Chemical Ecology 22, 1735-1766.

Raguso, R. A., Pichersky, E., 1995. Floral volatiles from *Clarkia breweri* and *C. concinna* (Onagraceae): Recent evolution of floral scent and moth pollination. Plant Systematics and Evolution 194, 55-67.

Robertson, J. L., Wyatt, R., 1990. Evidence for pollination ecotypes in the yellow-fringed orchid, *Platanthera ciliaris*. Evolution 44, 121-133.

Schiestl, F. P., 2015. Ecology and evolution of floral volatile-mediated information transfer in plants. New Phytologist 206, 571-577.

Schiestl, F. P., Schluter, P. M., 2009. Floral Isolation, Specialized Pollination, and Pollinator Behavior in Orchids. Annual Review of Entomology 54, 425-446.

Schlechter, R., 1895. Contributions to the knowledge of new and critical orchids from South Africa. Botansche Jahrbucher fur Systematik, Pflanzengeschichte und Pflanzengeographie 20.

Schlechter, R., 1915. Contributions to the Flora of Africa XLV. Botansche Jahrbucher fur Systematik, Pflanzengeschichte und Pflanzengeographie 53, 527-529.

Schmid, S., Schmid, V., Zillikens, A., Harter-Marques, B., Steiner, J., 2011. Bimodal pollination system of the bromeliad *Aechmea nudicaulis* involving hummingbirds and bees. Plant Biology 13, 41-50.

Shuttleworth, A., Johnson, S. D., 2008. Bimodal pollination by wasps and beetles in the African milkweed *Xysmalobium undulatum*. Biotropica 40, 568-574.

Shuttleworth, A., Johnson, S. D., 2020. Using two confluent capillary columns for improved gas chromatography-electroantennographic detection (GC-EAD). Entomologia Experimentalis et Applicata 168, 191-197.

Shuttleworth, A., Johnson, S. D., Jürgens, A., 2017. Entering through the narrow gate: a morphological filter explains specialized pollination of a carrion-scented stapeliad. Flora 232, 92-103.

Steenhuisen, S.-L., Raguso, R., Johnson, S., 2012. Floral scent in bird-and beetle-pollinated *Protea* species (Proteaceae): Chemistry, emission rates and function. Phytochemistry 84, 78-87.

Summerhayes, V., 1966. African orchids: XXX. Kew Bulletin 20, 165-199.

Summerhayes, V. S., 1931. African orchids, II. Bulletin of Miscellaneous Information (Royal Botanic Gardens, Kew) 1931, 378-390.

Sun, X.-L., Wang, G.-C., Gao, Y., Zhang, X.-Z., Xin, Z.-J., Chen, Z.-M., 2014. Volatiles emitted from tea plants infested by Ectropis obliqua larvae are attractive to conspecific moths. Journal of Chemical Ecology 40, 1080-1089.

Theis, N., Lerdau, M., Raguso, R. A., 2007. The challenge of attracting pollinators while evading floral herbivores: patterns of fragrance emission in *Cirsium arvense* and *Cirsium repandum* (Asteraceae). International Journal of Plant Sciences 168, 587-601.

Van der Niet, T., Jürgens, A., Johnson, S., 2015. Is the timing of scent emission correlated with insect visitor activity and pollination in long-spurred *Satyrium* species? Plant Biology 17, 226-237.

Van der Niet, T., Liltved, W. R., Oliver, E. G. H., 2009. *Satyrium situsanguinum* (Orchidaceae): A new species from the Western Cape, South Africa. South African Journal of Botany 75, 22-26.

Van der Niet, T., Linder, H., 2008. Dealing with incongruence in the quest for the species tree: a case study from the orchid genus *Satyrium*. Molecular Phylogenetics and Evolution 47, 154-174.

Van der Niet, T., Linder, H., Bytebier, B., Bellstedt, D. U., 2005. Molecular Markers Reject Monophyly of the Subgenera of *Satyrium* (Orchidaceae). Systematic Biology 30, 263-274.

Van der Niet, T., Pirie, M. D., Shuttleworth, A., Johnson, S. D., Midgley, J. J., 2014. Do pollinator distributions underlie the evolution of pollination ecotypes in the Cape shrub *Erica plukenetii*? Annals of Botany 113, 301-315.

Violle, C., Navas, M. L., Vile, D., Kazakou, E., Fortunel, C., Hummel, I., Garnier, E., 2007. Let the concept of trait be functional! Oikos 116, 882-892.

Waser, N. M., Campbell, D. R. 2004. Ecological speciation in flowering plants. Pages 264-277.*in* U. Dieckmann, Doebeli, M., Metz, J. A. J., Tautz, D., eds. Adaptive speciation. Cambridge University Press, Cambridge.

Woodcock, T. S., Larson, B. M., Kevan, P. G., Inouye, D. W., Lunau, K., 2014. Flies and flowers II: Floral attractants and rewards. Journal of Pollination Ecology 12, 63-94.

Zhang, Q.-H., Schlyter, F., Anderson, P., 1999. Green leaf volatiles interrupt pheromone response of spruce bark beetle, *Ips typographus*. Journal of Chemical Ecology 25, 2847-2861.

CHAPTER 4 : *SATYRIUM BASUTORUM* (ORCHIDACEAE): A NEW SPECIES FROM THE DRAKENSBERG ALPINE CENTRE, SOUTHERN AFRICA

4.1 Abstract

Satyrium basutorum is described as a new species from the Drakensberg Alpine Centre in South Africa. It is recognised by its relatively short, often very densely packed inflorescence, which does not extend far above ground level, and by its cream-white relatively short-spurred flowers with distinct light to dark purple lines on sepals and petals.

4.2 Introduction

Satyrium Sw. is an orchid genus that is characterised by its twin-spurred, non-resupinate flowers (Kurzweil and Linder, 1999). The genus comprises 93 species (Hall, 1982; Van der Niet et al., 2005; Van der Niet and Cribb, 2006), which have a wide distribution throughout continental Africa, Madagascar and with a few species in Asia (Hall, 1982; Van der Niet et al., 2005; Van der Niet and Cribb, 2006). Major hotspots for the genus include the Cape Floristic Region (CFR), which is known for being a hotspot for numerous plant genera (Linder, 2003; Verboom et al., 2009a; Verboom et al., 2009b), with further radiations in *Satyrium* occurring along the Drakensberg Range into Limpopo and Mpumalanga in southern Africa, as well as in the grasslands of South-Central, and Eastern Africa, particularly in the Mountain Ranges along the Rift Valley (Hall, 1982; Johnson et al., 2011).

Satyrium neglectum Schltr. is considered a highly variable species complex, and comprises two subspecies: *S. neglectum* ssp. *neglectum* and *S. neglectum* ssp. *woodii* (Hall, 1982). The species is known to occur throughout southern and eastern Africa, with a distribution which occurs from the southern parts of the Drakensberg in the Eastern Cape, up into the Nyika Plateau in Malawi and Tanzania (Hall, 1982). Intra-specific morphological variation in ssp. *neglectum* led to the recognition of two forms, separated by distribution in differing elevations. The high-elevation form in southern Africa was linked to var. *brevicalcar* Summerh. based on similarity in spur length (Summerhayes, 1966; Hall, 1982). Variation in the morphology and preliminary phylogenetic evidence (Chapter 2) suggested that the South African populations

of the high-elevation form of ssp. *neglectum* represent an independent species, which necessitated a reinvestigation into taxonomy of the species complex.

The morphological analyses, scored from herbarium and preserved flowers, expanded phylogenetic findings of this thesis (Chapter 2), as well as ecological divergence in scent composition and pollination system (Chapter 3), supports the separation of South African populations of the high-elevation form from ssp. *neglectum*. The original description of the species (Hall, 1982), exclusively utilized herbarium material and did not explicitly consider the geographical origin of accessions within his analysis, the importance of ecological divergence in floral traits such as spur length or floral scent composition, or a phylogenetic perspective. The measurement of traits of preserved flowers and re-examination of herbarium material found that the high-elevation form is clearly morphologically distinct from the low-elevation form and from ssp. *woodii*. Additional ecological separation in scent composition revealed that the high-elevation form (moth pollination) is dominated by compounds associated with a different pollination system than either the low-elevation form (butterfly pollination) or ssp. *woodii* (bird pollination). Furthermore, phylogenetic analyses demonstrated that the high-elevation form is a strongly supported monophyletic clade, separate from the low-elevation form and from ssp. *woodii*, despite incongruence between plastid and nuclear topologies. Here, the high-elevation form is described as a new species in *Satyrium* from Drakensberg Mountain Centre.

4.3 Species Treatment

Satyrium basutorum Rule and Van der Niet.

TYPE ----

Eastern Cape Province, 3028, Peak of Naude's Nek Pass. ≥ 2200 m.

Rule 3. M.J. Rule, with Dr. T. Van der Niet. Collected on 9 February 2020. Bews Herbarium (NU), University of Kwa-Zulu Natal.

Terrestrial herbaceous plants. ***Vegetative stem*** erect to sub-erect, 80-250 mm tall, covered by several leaf sheaths. ***Tubers*** 1-2, ovoid. Glabrous. ***Leaves*** 2, basal, spreading but not adpressed to the ground, lanceolate, leaf point acute, entire, smooth, leathery, 64-86 × 14-35 mm, one generally larger than the other; Leaf ***sheaths*** 2-4, 3.2-11 mm long, lanceolate, sharply pointed, loosely clasping the stem. ***Inflorescence*** 47-141 mm long, sometimes slender, sometimes thickened; densely flowered, 6-34-flowered; bracts reflexed at roughly half their length, 3-11 mm × 5-11 mm, linear, sharply pointed, smooth, margins ciliate. ***Flowers*** non-resupinate, cream-white with distinct light to dark purple lines on sepals and petals. Semi-transparent. Sweetly scented. ***Ovary*** 5-8 mm long, longitudinally ridged, smooth. ***Sepals and lateral petals*** linear, slightly spathulate, flared at tips. ***Lateral sepals*** spreading, projecting at a 90° angle from galea opening, fused with median sepal and lateral petals for ⅕ of their length; the free part 5-8 × 1.7-2.6 mm. ***Median sepal*** projecting forward for half its length, then reflexing downward, the free part 5-8.4 × 1.6-2.7 mm. ***Lateral petals*** projecting forward for ± half their length, then sharply reflexing downward, curved, the free part 2-8×1.8- 3 mm. ***Labellum*** galeate, aperture 1.2 - 3.6 mm wide and 0.5-1.0 mm high, forward facing, hooded, apical flap prominent, 1.71–3.49 × 1.1-3.0 mm, erect, reflexed at 90° to galea aperture, rounded, slightly square, margins entire, apex has slight indentation in centre; ***spurs*** 6.88-11.8 mm long, projecting along ovary. ***Gynostemium***: ***column part*** 2.3-6.3 mm long, slightly curved towards galea aperture. ***Stigma*** erect, 0.60-1.75 × 0.9-2.9 mm, triangular, slightly pointed apex. ***Rostellum*** long slightly rounded, sharply pointed, 1-2.3 × 1-1.9 mm, extending beyond the laterally placed, circular viscidia. ***Anther*** 0.8-1 × 0.6-0.8 mm.

4.4 Diagnostic characteristics and relationships

Satyrium basutorum Rule and Van der Niet. can be identified by the relatively short, often very densely packed inflorescence (Figure 4.1). The flowers of *S. basutorum* are sweetly scented and semi-transparent. Flower colour varies in intensity, but the flowers are always white with distinct purple tinges along the middle of the sepals and petals. The sepals and petals of *S. basutorm* are spreading, often perpendicular to galea aperture. The spurs extend roughly half their length beyond the ovary, and the rostellum has a distinct pointed beak with lateral viscidia.

Satyrium neglectum ssp. *neglectum* (Schltr.) A.V. Hall. differs from *S. basutorum* in having a substantially longer inflorescence, with larger spaces in between flowers, and longer leaves. The flowers of *S. neglectum* ssp. *neglectum* differ in that they are generally smaller in length, wholly light-dark pink, with no discernible scent. The petals and sepals of *S. neglectum* ssp. *neglectum* are more intensely reflexed then those of *S. basutorum*, and are much thicker. The spurs of ssp. *neglectum* are generally longer in length. The rostellum of *S. neglectum* ssp. *neglectum* is flared towards its apex.

Satyrium neglectum ssp. *woodii* (Schltr.) A.V. Hall, differs from *S. basutorum* in having a much longer inflorescence and flowering stem, more so than is found in *S. neglectum* ssp. *neglectum*. The flowers of *S. neglectum* ssp. *woodii* are much more robust and larger than either of the above two concepts. Flowers of ssp. *woodii* also have a different orientation, appearing slightly rotated downwards, than flowers of either *S. basutorum* or ssp. *neglectum*. The petals and sepals of *S. neglectum* ssp. *woodii* usually occur in shades of orange and red.

Some forms of the highly variable *Satyrium longicauda* Lindl. forms, which are widespread in South Africa and further north into tropical eastern African countries. *S. longicauda* closely resembles *S. basutorum* in form, often occurring together and initially might be difficult to distinguish. The most apparent difference between the two, is the presence of hair-like structures on the apical lip of the labellum in *S. longicauda*, a feature which is absent in *S. basutorum.* While the overall size of the inflorescence and flowers are similar, the flowers of *S. basutorum* are often placed in a congested manner along the inflorescence, being tightly packed together, whereas the flowers of *S. longicauda* generally have a much larger space between flowers on the inflorescence. Furthermore, where the two species coexist, the flowers of *S. longicauda* differ in colour, with *S. longicauda* only having white or slightly tinged petals and sepals, with no purple-pink floral tinges along the centre. The spur length (which ranges

between 20 - 40 mm) of most forms of *S. longicauda* are much longer in length (Castañeda-Zárate et al., 2021) than those of *S. basutorm*, but some forms have spurs that are of equal length. In addition, there are differences in the structure of the rostellum apex, being more elliptic in *S. longicauda* and scent composition between *S. basutorum* and *S. longicauda* (Castañeda-Zárate et al., 2021). The scent composition of *S. longicauda* is dominated by aromatic compounds associated with moth pollination, in particular (E)-cinnamyl alcohol (Castañeda-Zárate et al., 2021).

4.5 Habitat

Satyrium basutorum occurs in areas of high elevation (always above c. 2200m above sea level) at sites along the Drakensberg Mountain Range. Individuals were often growing in sodden basalt soil or along rocky outcrops. Plants grow either in wet grassland, or among shrubs in basalt gravel, usually close to the escarpment edge.

4.6 Distribution

S. basutorum is known from a few localities along the Drakensberg escarpment, ranging from Mont Aux-Sources in the north to Naude's Nek in the south, but possibly extending further south in high-elevation mountains in the Eastern Cape, with reference to Naudes Nek, on basalt soil. *S. basutorum* is one of three *Satyrium* species which occur along the escarpment, the other two being *S. microrrhynchum* (Johnson et al., 2007), and some forms of *S. longicauda* (Castañeda-Zárate et al., 2021).

Figure 4.1: *Satyrium basutorum* in habitat (Photo S.D. Johnson and M.J. Rule)

4.7 Etymology

Greek: *Satyrium* = in reference to double spurred labellum, and satyrs from Greek mythology, and Latin: *basutorum* = in honour of Basotho. The species named after the locality it was first described, Lesotho.

4.8 Flowering time

Based on herbarium records, *S. basutorum* flowers in the early months of the year. Flowering starts in the beginning of January continues towards mid-late February.

4.9 Conservation status

S. basutorum is known from only a few areas of high-elevation in southern Africa, particularly in the Drakensberg Mountain Centre. According to the IUCN (2001) Red List and Criteria, the species, *S. basutorum*, meets the following criteria from currently known information: (1) it is estimated to occupy less than 20,000 km^2, (1) is known to occur at less than 10 localities and may be considered (3) fragmented, occurring only in areas of high-elevation within the Drakensberg Mountain Centre, with known populations having numbers ranging between 14-40. This coupled with the complete loss of the population at Sani Pass, may suggest the species is currently vulnerable. However, this is based on collector information recorded in herbarium records, which were limited in number. The distribution of this species in areas of high-elevation along the Drakensberg escarpment may explain its rarity in herbarium collections. Thus, the rarity of the concept may reflect under-collection due to the inaccessibility of populations. Despite this apparent underrepresentation in herbarium collections, the species may however be subject to encroachment from alien invasive species, and extensive cattle-grazing along the Drakensberg escarpment (O'Connor, 2005; Carbutt, 2012). As the true extent of the species distribution and population numbers are underrepresented in herbarium collections, *S. basutorum* should thus be categorised as Vulnerable (V), requiring additional surveys as the exact number of populations and their sizes.

4.10 References

Carbutt, C., 2012. The emerging invasive alien plants of the Drakensberg Alpine Centre, southern Africa. Bothalia 42, 71-85.

Castañeda-Zárate, M., Johnson, S. D., van der Niet, T., 2021. Food Reward Chemistry Explains a Novel Pollinator Shift and Vestigialization of Long Floral Spurs in an Orchid. Current Biology 31, 238-246. e237.

Hall, A. V. 1982. A Revision of the Southern African Species of *Satyrium*. Bolus Herbarium, Rondebosch, Republic of South Africa.

IUCN. 2001. IUCN Red List categories and criteria. IUCN.

Johnson, S. D., Ellis, A., Dotterl, S., 2007. Specialization for pollination by beetles and wasps: The role of lollipop hairs and fragrance in *Satyrium microrrhynchum* (Orchidaceae). American Journal of Botany 94, 47-55.

Johnson, S. D., Peter, C. I., Ellis, A. G., Boberg, E., Botes, C., Van der Niet, T., 2011. Diverse pollination systems of the twin-spurred orchid genus Satyrium in African grasslands. Plant Systematics and Evolution 292, 95-103.

Kurzweil, H., Linder, H. P., 1999. A phylogenetic analysis of the genus *Satyrium* (Orchidaceae). Beitraege zur Biologie der Pflanzen 71, 101-181.

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

O'Connor, T., 2005. Influence of land use on plant community composition and diversity in Highland Sourveld grassland in the southern Drakensberg, South Africa. Journal of Applied Ecology 42, 975-988.

Summerhayes, V., 1966. African orchids: XXX. Kew Bulletin 20, 165-199.

Van der Niet, T., Cribb, P., 2006. Additions to the orchid flora of Angola, Tanzania and Zimbabwe. Kew Bulletin, 261-264.

Van der Niet, T., Linder, H. P., Bytebier, B., Bellstedt, D. U., 2005. Molecular markers reject monophyly of the subgenera of *Satyrium* (Orchidaceae). Systematic Botany 30, 263-274.

Verboom, G. A., Archibald, J. K., Bakker, F. T., Bellstedt, D. U., Conrad, F., Dreyer, L. L., Forest, F., Galley, C., Goldblatt, P., Henning, J. F., 2009a. Origin and diversification of the Greater Cape flora: ancient species repository, hot-bed of recent radiation, or both? Molecular Phylogenetics and Evolution 51, 44-53.

Verboom, G. A., Dreyer, L. L., Savolainen, V., 2009b. Understanding the origins and evolution of the world's biodiversity hotspots: The biota of the African 'Cape Floristic Region' as a case study Preface. Molecular Phylogenetics and Evolution 51, 1-4.

CHAPTER 5 : CONCLUSIONS AND RECOMMENDATIONS FOR FURTHER RESEARCH

5.1 Outline

Conventional herbarium-based taxonomy has relied on the use of distinctive morphological floral and vegetative traits to delimit species boundaries. However, the exclusive use of morphological studies can lead to under- or over-estimation of biodiversity. Limited herbarium records may misrepresent continuous variation as discontinuous in taxa, thereby leading to an overestimation of species diversity, and is often inaccurate in the use of diagnostic traits that are, in fact, homoplasious, arising from convergent evolution. The aim of this study was to investigate the systematics and scent chemistry of the *Satyrium neglectum* species complex. This study highlights the potential of a multidisciplinary study that incorporates phylogenetic evidence as well as an analysis of chemical traits for taxonomic revisions, with the ultimate goal of attaining more accurate estimates of taxa and their relationships.

In Chapter 2, I investigated the species boundaries of the *Satyrium neglectum* species complex using a combination of traditional morphological techniques and phylogenetic analyses. Similar to numerous other plant species, *S. neglectum* has undergone extensive taxonomic changes with many synonyms now combined into a singular taxonomic concept (Hall, 1982). Many species revisions and descriptions relied on the use of morphological characters scored exclusively from herbarium records to examine similarities between taxa and consolidate superfluous species concepts into synonymy. While herbaria provide a critical source of information, herbarium collections are not without their limitations (Bebber et al., 2010; Parnell et al., 2013), as the act of preserving specimens for herbarium storage often leads to the distortion and loss of traits which are vital in species delimitation, especially in orchids, where the 3-D floral structure is lost (Gentry, 1989; Parnell et al., 2013).

I provided a comparative morphological analysis comparing specimens from taxa that were relegated into synonymy within the *Satyrium neglectum* species complex. The most recent revision of the genus relied solely on herbarium records, and did not explicitly define geographical origins of samples, which limits the opportunity for examination of morphological variation from a geographic perspective (Hall, 1982). This study found that the South African high-elevation form is morphologically distinct and forms an independent,

monophyletic lineage, separate from all other accessions within the *S. neglectum* complex. The separation between taxa, while evident in herbarium records, was more clearly distinct in records scored from ethanol-preserved flowers. This study thus emphasises the importance of including fresh material, as distortion of floral structures in mechanical pressing and underrepresentation of herbarium material may lead to inaccurate delimitation. Reinstatement of taxonomic groups, or description of new ones, requires both an extensive morphological and phylogenetic analysis, and this chapter illustrated that complementing herbarium-based taxonomy with phylogenetic analyses can affect the number of taxa recognized, and lead to an increase of recognised biodiversity.

In Chapter 3, I investigated the floral scent chemistry and its relation to ecology and pollination in the *S. neglectum* species complex. Diversity in floral traits may be the result of pollinator shifts, and can lead to divergence and speciation (Stebbins, 1970; Johnson, 1997; Van der Niet et al., 2014). Despite this, many floral traits are not included in taxonomic revisions, or cannot be included in herbarium records on which these revisions are based. Differences in floral chemistry in relation to pollination systems may provide an additional facet, alongside morphology and phylogenetic analyses, in the circumspection of taxonomic groups, which are difficult to distinguish. Scent plays, a key role in numerous pollination syndromes (Proctor et al., 1996; Dobson, 2006; Knudsen et al., 2006; Van der Niet et al., 2014), and variation in floral traits suggests a shift in pollination systems (Johnson, 1997; Van der Niet et al., 2014).

I provided a comparative analysis of floral scent composition, emission rates and differentiation between dominant compounds in the *S. neglectum* species complex. My study found that scent composition and emission rates differ between the high- and low-elevation form and ssp. *woodii* which provides an additional trait that reflects their separation in the phylogeny.

Differences between scent compounds are also largely reflective of differences in pollination systems. Lack of scent and reduced emission rates of floral scent in ssp. *woodii* reflect bird pollination as observed in previous studies (Faegri and van der Pijl, 1979; Dobson, 2006; Van der Niet et al., 2015a; Johnson and Van der Niet, 2019). Furthermore, while pollinator observations of the low-elevation form did not confirm butterfly pollination, the presence of compounds, which are largely associated with butterfly pollination, were present in the scent bouquet of the low-elevation form (Honda et al., 1998; Huber et al., 2005; Dobson, 2006). Dominant compounds in the scent bouquet of the high-elevation form, are closely associated

with moth-pollination (Huber et al., 2005; Van der Niet et al., 2015b), in addition, to observations and recorded electroantennographic responses to these two compounds in two settling moths from Naudes Nek. Settling moth visitation contradicts initial pollinator observations in Johnson et al. (2011), which revealed long-proboscid fly pollination, which is usually not associated with scent (Goldblatt and Manning, 2000).

Finally, cumulative evidence from a multidisciplinary background, including phylogenetic, morphological and ecological warranted the need to describe the high-elevation form as a new species: *Satyrium basutorum* Van der Niet and Rule *(*Chapter 4*).*

5.2 Conclusions and Recommendations for Further Study

In this study, I established how complementing exclusive use of herbarium based morphological analyses with phylogenetic analyses and quantification of floral scent chemistry can be used for species delimitation. Through this integrative approach, a new species has been described, *Satyrium basutorum* (Chapter 4). The concept of species delimitation has long been debated, and concepts, which sought to define what a species is, have been based on multiple disciplines, including ecological, morphological and phylogenetic ones. The findings of the morphological analysis, expansion of accessions in the phylogeny of this thesis (Chapter 2), and evidence for ecological divergence in scent composition and pollination system (Chapter 3), supports the separation of the high-elevation form from ssp. *neglectum*. The original revision of *S. neglectum* (Hall, 1982), ascribed morphological variation in floral traits as insufficient for delimitation, despite their close association with floral divergence and speciation (Stebbins, 1970; Johnson, 1997).

Further studies in this system should look at the possibility of a reinstatement of two synonyms of the current *Satyrium neglectum* ssp. *neglectum* and ssp. *woodii*: var. *brevicalcar* and *S. sceptrum* respectively. While this thesis concluded that var. *brevicalcar* and *S. sceptrum* remain as synonyms within their original taxonomic grouping, this was based on the available evidence. Pollination and additional ecological studies, and the examination of fresh material and DNA accessions from source material may provide further insight into their taxonomic position. In addition, further work in the system should include more detailed pollination observations on the high-elevation form with the aim of determining the pollination efficiency of both moths and long-proboscid flies, and identifying behavioural responses to scent

compounds. Choice experiments using dominant scent compounds can be used to ascertain their effect on pollinator's behaviour. Furthermore, pollinator observations of the high-elevation form were particularly difficult, owing to hazardous weather conditions making the population inaccessible. Camera traps offer promise to provide further proof of settling moth pollination, and allow observations to occur when being otherwise impossible. At the time of submission of this thesis, camera trapping in the Drakensberg by T van der Niet and SD Johnson was providing valuable new records of settling moth pollination of the proposed taxon *Satyrium basutorum.*

Figure 5.1: Settling moth visitation in *S. basutorum* as recorded by camera trapping in the Drakensberg (T. van der Niet, Ruth Cozien and S.D. Johnson)

5.3 References

Bebber, D. P., Carine, M. A., Wood, J. R., Wortley, A. H., Harris, D. J., Prance, G. T., Davidse, G., Paige, J., Pennington, T. D., Robson, N. K., 2010. Herbaria are a major frontier for species discovery. Proceedings of the National Academy of Sciences 107, 22169-22171.

Dobson, H. 2006. Relationship between floral fragrance composition and type of pollinator. Pages 147-198.In D. N , E, P., eds. Biology of Floral Scent. CRC Press, Boca Raton: .

Faegri, K., van der Pijl, L. 1979. The Principles of Pollination Ecology. Pergamon, Oxford.

Gentry, A., 1989. Herbarium taxonomy versus field knowledge. Is there an attainable solution? Flora Malesiana Bulletin 10, 31-35.

Goldblatt, P., Manning, J. C., 2000. The long-proboscid fly pollination system in southern Africa. Annals of the Missouri Botanical Garden 87, 146-170.

Hall, A. V. 1982. A Revision of the Southern African Species of *Satyrium*. Bolus Herbarium, Rondebosch, Republic of South Africa.

Honda, K., Ômura, H., Hayashi, N., 1998. Identification of floral volatiles from *Ligustrum japonicum* that stimulate flower-visiting by Cabbage butterfly, *Pieris rapae*. Journal of Chemical Ecology 24, 2167-2180.

Huber, F. K., Kaiser, R., Sauter, W., Schiestl, F. P., 2005. Floral scent emission and pollinator attraction in two species of Gymnadenia (Orchidaceae). Oecologia 142, 564-575.

Johnson, S., 1997. Pollination ecotypes of *Satyrium hallackii* (Orchidaceae) in South Africa. Botanical Journal of the Linnean Society 123, 225-235.

Johnson, S. D., Peter, C. I., Ellis, A. G., Boberg, E., Botes, C., Van der Niet, T., 2011. Diverse pollination systems of the twin-spurred orchid genus *Satyrium* in African grasslands. Plant Systematics and Evolution 292, 95-103.

Johnson, S. D., Van der Niet, T., 2019. Bird pollination in an African *Satyrium* (Orchidaceae) confirmed by camera traps and selective exclusion experiments. Plant Systematics and Evolution 305, 477-484.

Knudsen, J. T., Eriksson, R., Gershenzon, J., Stahl, B., 2006. Diversity and distribution of floral scent. Botanical Review 72, 1-120.

Parnell, J., Rich, T., McVeigh, A., Lim, A., Quigley, S., Morris, D., Wong, Z., 2013. The effect of preservation methods on plant morphology. Taxon 62, 1259-1265.

Proctor, M., Yeo, P., Lack, A. 1996. The Natural History of Pollination. Collins, London.

Stebbins, G. L., 1970. Adaptive radiation of reproductive characteristics in angiosperms. I. Pollination mechanisms. Annual Review of Ecology and Systematics 1, 307-326.

Van der Niet, T., Cozien, R. J., Johnson, S. D., 2015a. Experimental evidence for specialized bird pollination in the endangered South African orchid *Satyrium rhodanthum* and analysis of associated floral traits. Botanical Journal of the Linnean Society 177, 141-150.

Van der Niet, T., Jürgens, A., Johnson, S., 2015b. Is the timing of scent emission correlated with insect visitor activity and pollination in long-spurred *Satyrium* species? Plant Biology 17, 226-237.

Van der Niet, T., Pirie, M. D., Shuttleworth, A., Johnson, S. D., Midgley, J. J., 2014. Do pollinator distributions underlie the evolution of pollination ecotypes in the Cape shrub *Erica plukenetii*? Annals of Botany 113, 301-315.

APPENDIX A: Supplementary Tables

Table S1: Mean ± SD of floral and vegetative (all in cm) traits measured in Herbarium samples of the *Satyrium neglectum* complex. Ratios represent length divided by width.

Trait	**Mean ± SD**					
	ssp. *neglectum*				ssp. *woodii*	
	High-form	Low-form	var. *brevicalcar*	*S. acutirostrum*	*S. sceptrum*	*S. woodii*
Inflorescence Length	9.27 ± 2.56	19.57 ± 7.91	11.53 ± 2.71	11.78 ± 4.28	13.74 ± 5.81	10.05 ± 2.74
Vegetative Stem Length	16.71 ± 5.71	39.19 ± 13.64	29.25 ± 12.17	50.11 ± 13.05	42.21 ± 19.25	48.25 ± 9.55
Number of Flowers	21.22 ± 7.54	44.39 ± 20.9	21.33 ± 7.28	24.42 ± 10.62	29.59 ± 12.85	21.83 ± 8.66
Spur Length	0.89 ± 0.16	1.36 ± 0.24	0.88 ± 0.24	1.28 ± 0.36	1.15 ± 0.16	1.78 ± 0.33
Median Sepal Length	0.61 ± 0.15	0.42 ± 0.13	0.37 ± 0.07	0.68 ± 0.14	0.53 ± 0.15	0.98 ± 0.59
Median Sepal Width	0.14 ± 0.05	0.14 ± 0.04	0.11 ± 0.04	0.2 ± 0.04	0.17 ± 0.01	0.17 ± 0.02
Median Sepal Ratio	4.67 ± 1.53	3.03 ± 0.98	3.36 ± 0.6	3.46 ± 1	3.06 ± 0.79	5.88 ± 3.95
Lateral Sepal Length	0.6 ± 0.09	0.48 ± 0.09	0.41 ± 0.06	0.66 ± 0.14	0.55 ± 0.07	0.84 ± 0.12
Lateral Sepal Width	0.18 ± 0.03	0.19 ± 0.04	0.17 ± 0.04	0.28 ± 0.06	0.24 ± 0.03	0.32 ± 0.04
Lateral Sepal Ratio	3.34 ± 0.58	2.58 ± 0.65	2.55 ± 0.75	2.39 ± 0.42	2.34 ± 0.29	2.71 ± 0.51
Petal Length	0.54 ± 0.11	0.43 ± 0.09	0.35 ± 0.07	0.59 ± 0.09	0.51 ± 0.11	0.81 ± 0.21
Petal Width	0.15 ± 0.03	0.14 ± 0.03	0.13 ± 0.03	0.24 ± 0.05	0.19 ± 0.04	0.25 ± 0.09
Petal Ratio	3.8 ± 1.17	3.1 ± 0.75	2.69 ± 0.53	2.5 ± 0.55	2.73 ± 0.71	3.64 ± 1.49
Lip Flap Length	0.19 ± 0.04	0.15 ± 0.03	0.13 ± 0.03	0.19 ± 0.02	0.16 ± 0.03	0.18 ± 0.06
Lip Flap Width	0.2 ± 0.05	0.15 ± 0.03	0.16 ± 0.02	0.2 ± 0.04	0.17 ± 0.03	0.21 ± 0.05
Lip Flap Ratio	1 ± 0.4	1.02 ± 0.25	0.82 ± 0.11	0.94 ± 0.22	0.99 ± 0.22	0.89 ± 0.31

Table S2: Mean ± SD of floral (mm) traits measured in the preserved flowers of *S. neglectum* ssp. *neglectum*

Trait	**Locality and Form**						
		High-form			Low-form		ssp. *woodii*
	Mt. Aux Sources	Sani Pass	Naudes Nek	Van Reenen's Pass	Garden Castle	Bushman's Nek	Malcom's Farm
Spur Length	8.89 ± 1.16	8.89 ± 0.98	9.4 ± 1.08	12.37 ± 1.4	10.69 ± 1.26	10.23 ± 1.14	14.94 ± 1.59
Median Sepal Length	7.51 ± 0.64	6.38 ± 0.81	6.97 ± 0.55	4.08 ± 0.46	4.61 ± 0.57	4.58 ± 0.67	8.68 ± 1.09
Median Sepal Width	2.07 ± 0.26	2.04 ± 0.25	2.03 ± 0.15	1.46 ± 0.2	1.95 ± 0.2	1.84 ± 0.32	2.65 ± 0.31
Lateral Sepal Length	7.14 ± 0.52	6.26 ± 0.53	6.54 ± 0.53	5.15 ± 0.54	5.31 ± 0.57	5.21 ± 0.55	8.72 ± 0.99
Lateral Sepal Width	2.27 ± 0.21	2.44 ± 0.81	2.17 ± 0.23	2.23 ± 0.34	2.58 ± 0.27	2.34 ± 0.27	3.94 ± 0.61
Petal Length	6.9 ± 0.63	5.49 ± 1.08	6.48 ± 0.51	4.56 ± 0.26	4.36 ± 0.72	4.82 ± 0.62	9.83 ± 1.29
Petal Width	2.38 ± 0.36	2.35 ± 0.32	2.38 ± 0.22	1.54 ± 0.21	1.68 ± 0.3	1.63 ± 0.24	3.83 ± 0.35
Apical Flap Length	2.16 ± 0.41	2.07 ± 0.27	1.99 ± 0.32	1.65 ± 0.38	1.91 ± 0.26	1.77 ± 0.31	2.14 ± 0.34
Apical Flap Width	2.38 ± 0.34	2.49 ± 0.17	2.4 ± 0.29	1.99 ± 0.23	2.06 ± 0.35	2.01 ± 0.28	3.47 ± 0.83
Ovary Length	6.63 ± 0.98	6.33 ± 0.92	5.64 ± 1.03	9.19 ± 1.03	8.77 ± 1.24	8.36 ± 2.4	10.18 ± 2.25
Column Length	4.01 ± 0.71	3.51 ± 0.37	3.12 ± 0.31	4.91 ± 0.13	4.91 ± 0.63	4.46 ± 0.94	8.19 ± 1.48
Stigma Flap Length	1.23 ± 0.14	1.09 ± 0.2	0.95 ± 0.2	1.05 ± 0.12	0.78 ± 0.22	0.88 ± 0.19	2.39 ± 0.44
Stigma Flap Width	2.03 ± 0.27	1.88 ± 0.17	1.9 ± 0.3	1.54 ± 0.35	1.54 ± 0.22	1.35 ± 0.18	3.33 ± 0.55
Rostellum Length	1.67 ± 0.25	1.63 ± 0.39	1.49 ± 0.2	1.31 ± 0.31	1.46 ± 0.28	1.46 ± 0.18	3.71 ± 0.43
Rostellum Width	1.37 ± 0.17	1.23 ± 0.15	1.4 ± 0.14	0.98 ± 0.05	0.99 ± 0.14	1.21 ± 0.19	2.09 ± 0.34

Table S3: Herbarium Records of *S. neglectum* species complex used in this study

Herbarium	Collector	Collector Number	Country	Elevation (m)
	S. acutirostrum			
K	Ball, J. S.	791	TZ	2094
K	Bidgood, S. & Congdon T.C.E.	123	TZ	2600
K	Cribb, P., Grey-Wilson, C. & Mwasumbi, L.	10755	TZ	2304
K	Falk, K.G.P.	41	TZ	
K	Holmes, W.D.	208	MW	2094
K	Hopper, Townsend & Nicholson	819	TZ	2200
K	Lovett, J.	2017	TZ	1830
K	Stolz, A.	2528	TZ	
K	Suleimman H, Fundi MJ	21	TZ	2400
K	Wild, H.	4929	ZI	1963
K	Williamson, G. & Odgers A.	267	MW	
	S. neglectum var. brevicalcar			
K	Ball, J.S.	792	TZ	
K	Richards, H.M.	14298		
K	Semsei, S.R.	1694	TZ	2200
K	St Claire-Thompson	683	KE	
K	Stolz, A.	2456		
K	Stolz, A.	1188		1600
	***S. neglectum* ssp. *neglectum* - High**			
NU	Beverly, A.C.	460	LS	2550
NU	Boberg, E.	22	SA	
NU	Boberg, E.	24	SA	
NU	Boberg, E.	23	SA	
NU	Edwards, D.	1167	SA	3049
NU	Richardson	221	LS	2500
NU	Schmitz, M.	8929	LS	2000
NU	Sterwart, J.	1952	SA	2438
NU	Sterwart, J.	2207	LS	
NU	Stewart, J. & Manning, J.	2260	SA	2805
PRE	Bester, SP.	3621	SA	2500
PRE	Bester, SP.	3484	SA	2500
PRE	Herbst, N.	5293	LS	2612
PRE	Killick, D.J.B.	4533	LS	2700
PRE	Killick, D.J.B.	2320	LS	3000
PRE	Marais, W.	1375	SA	2500
PRE	Venter, S.	1565	-	2844
PRE	Venter, S.	2884	-	3000
PRE	Venter, S.	2876	-	2800
	***S. neglectum* ssp. *neglectum* - Low**			

K	Alle, D.J.	534	MW	2348
K	Bidgood, S. & Congdon, T.C.E.	125	TZ	2600
K	Brummit. R.K.	9123	MW	1720
K	Burtt, B.D.	4446	TZ	1982
K	Bytebier, B.	2518	TZ	2584
K	Cribb, P. & Grey-Wilson, C.	10608	TZ	1571
K	Cribb, P., Grey-Wilson, C. & Mwasumbi, L.	10754	TZ	2744
K	Gereau, R., Lovett, J. & Mtweve, J.	3089	TZ	2570
K	Grosvenor, R.K.	334	ZI	1677
K	Grosvenor, R.K. & Renz, J.	977	MW	1900
K	Grosvenor, R.K. & Renz, J.	1313	ZI	2000
K	Harris, T.	267	MQ	514
K	Lady Drewe	57826	ZI	1571
K	Leedal, G.	4829	TZ	2744
K	Lovett, J. & Congdon, T.C.E.	1842	TZ	2400
K	Philcox, D. and Leppard, M.J.	8917	ZI	762
K	Plowes, D.C.H.	2148	ZI	1982
K	Raal, P. & G.	1423	ZI	1616
K	Richards, H.M.	7700	TZ	2250
K	Richards, H.M.	14173	TZ	2100
K	Richards, H.M.	14147	TZ	2100
K	Rowe	291	NI	1623
K	Ruch, M.	1730	LS	1677
K	Schlr, Z.	988	SA	1524
K	Schlr, Z.	1000	SA	1524
K	Thulin, M. & Mhoro, B.	3033	ZI	534
NU	Acocks, J.P.H.	13956	SA	1159
NU	Bourquin	945	SA	1524
NU	Edwards, D.	567	SA	2073
NU	Gibson, J.	17	SA	1616
NU	Hillliard, O.M. & Burtt, B. L.	16269	SA	1524
NU	Hillliard, O.M. & Burtt, B. L.	18151	SA	1951
NU	Hillliard, O.M. & Burtt, B. L.	17501	SA	2134
NU	O'Conner, M. J.	329	SA	595
NU	Richards, H.M.	7643	TZ	2400
NU	Stewart, J.	1519	SA	1738
NU	Stewart, J.	2096	SA	2096
NU	Trace, C.	45	SA	1921
PRE	Bolus, H.	10976	SA	1555
PRE	Codd, L.E.	6397	SW	1494
PRE	Compton, R.H.	28545	SW	1524
PRE	Compton, R.H.	567	SW	1220
PRE	Compton, R.H.	27390	SW	1372
PRE	Compton, R.H.	25745	SW	1220
PRE	Devenish, N.J.	414	SA	1951

PRE	Galpin, E.E.	9881	SA	1829
PRE	Galpin, E.E.	11702	SA	1982
PRE	Galpin, E.E.	718	SW	1524
PRE	Germishuizen	48	SA	1047
PRE	Jacobsz, M.L.	1643	SA	1700
PRE	Killick	3850	SA	1707
PRE	Killick and Vahremeyer	3676	SA	1524
PRE	Killick and Vahremeyer	3786	SA	1982
PRE	Kluge, J.P.	2483	SA	1900
PRE	Lavranos, J.	15263	SA	1500
PRE	Nkuna & Mbatha	2092	SA	1427
PRE	Raal, P. & G.	1504	SA	1620
PRE	Rennie, M. A.	1298	SA	1829
PRE	Simon, B.K.	733	LS	1982
PRE	van der Merwe	22	SA	2134
PRE	Venter, S.	1500	SA	1970
PRE	West, O.	603	SA	2134
	S. sceptrum			
K	Bally	7411	KE	2173
K	Cribb, P., Grey-Wilson, C. & Mwasumbi, L.	11302	TZ	2100
K	Cuthbert, N.F.	30	TZ	
K	Davis, D.	2	KE	1963
K	Dgsone, W.G.	413	KE	1885
K	Glover, Gwynne & Samuel	1792	KE	1885
K	Graham, R.M.	1020	KE	2356
K	Graham, R.M.	1005	KE	2225
K	Kayomba, C.J.	326	TZ	1850
K	Lugard, E.J.	279	TZ	2251
K	Pierce, R.	2694	KE	1832
K	Richards, H.M.	8565	TZ	1650
K	Slade, H.	2	KE	2094
K	Taylor, P.	10400	TZ	1570
K	Tweedie, L. & Carrol, E.W.	622	KE	1309
K	Verdcourt	696	KE	2356
K	Ward, E.J.	1149	TZ	
	S. woodii			
K	Ward, E.J.	625	SA	
NU	O'Conner	110	SA	
PRE	Pegler, A.	242C	SA	
PRE	Rudatis, A.G.H.	751	SA	
PRE	Tyson	2087	SA	
PRE	Werdermann	1210	SA	

Table S4: List of New Accessions included in the phylogenetic analysis of this study with geographical origin, GenBank accession and abbreviation used in Fig. 2.10

Taxon	Abbreviation	Locality	trnL intron	trnL-trnF intergenic spacer	ITS	trnS-trnG intergenic spacer	matK
Satyrium neglectum ssp. *neglectum*	S. neglectum A1	Mt. Aux-Sources	MW345580	MW345566	MW345538	MW345595	MW345551
	S. neglectum A2	Mt. Aux-Sources	MW345581	MW345566	-	MW345596	MW345552
	S. neglectum B1	Bushman's Nek	MW345582	MW345567	MW345539	MW345597	MW345553
	S. neglectum B3	Bushman's Nek	MW345583	MW345568	MW345540	MW345598	MW345554
	S. neglectum G2	Graskop	MW345584	MW345569	MW345541	MW345599	MW345555
	S. neglectum G3	Graskop	MW345585	MW345570	MW345542	MW345600	MW345556
	S. neglectum N2	Naude's Nek	MW345586	MW345571	MW345543	MW345601	MW345557
	S. neglectum N3	Naude's Nek	MW345587	MW345572	MW345544	MW345602	MW345558
	S. neglectum Q2	Qacha's Nek	MW345588	MW345573	MW345545	MW345603	MW345559
	S. neglectum Q3	Qacha's Nek	MW345589	MW345574	-	MW345604	MW345560
	S. neglectum S1	Sani Pass	MW345590	MW345575	MW345546	MW345605	MW345561
	S. neglectum S2	Sani Pass	MW345591	MW345576	MW345547	MW345606	MW345562
	S. neglectum V1	Van Reenan's Pass	MW345592	MW345577	MW345548	MW345607	MW345563
	S. neglectum V2	Van Reenan's Pass	MW345593	MW345579	MW345549	MW345608	MW345564
Satyrium neglectum ssp. *woodii*	S. woodii		MW345594	MW345579	MW345550	MW345609	-

Table S5: Mean ± SD relative amounts (%) of floral scent compounds of the high- and low-elevation form of *Satyrium neglectum* ssp. *neglectum*. Compounds are listed according to their compound class and sorted by Kovats Retention Index (KRI) (Species localities are abbreviated as follows: G = Graskop, V = Van Reenan's Pass, S = Sani Pass, B = Bushman's Nek, Q = Qacha's Nek, A = Mt. Aux-Sources, N = Naude's Nek).

		Low- form						**High-form**		
		G	V	S	B	Q	A	S	N	N
Number of samples		3	4	4	4	2	4	4	2	4
Sampling time		N	D	N	D	D	D	D	D	N
Mean Number of Flowers		-	12.75 ± 6.45	37 ± 13.64	-	60 ± 19.8	-	16.25 ± 4.5	10 ± 2.83	14.5 ± 6.86
Mean total emission (µg)		1.47 ± 0.32	9.43 ± 3.19	1.42 ± 1.01	9.56 ± 5.24	15.52 ± 20.63	1.53 ± 0.93	4.3 ± 3.55	0.14 ± 0.16	0.28 ± 0.15
Mean total emission ($\mu g h^{-1}$)		3.53 ± 0.76	28.29 ± 9.56	3.41 ± 2.43	28.67 ± 15.73	37.25 ± 49.51	4.17 ± 2.52	11.72 ± 9.68	0.43 ± 0.47	0.68 ± 0.36
Mean total emission ($\mu g h^{-1}$/flower)		-	2480.12 ± 741.44	79.69 ± 46.59	-	512.54 ± 656.05	-	680.1 ± 439.19	38.18 ± 35.72	46.62 ± 4.85
Compound[1]	**KRI**									
Aliphatics										
Acids										
Acetic acid	1579	-	-	-	-	0.03 ± 0.05	-	0.2 ± 0.27	-	-
Butanoic acid	1613	0.003 ± 0.006	-	-	0.1 ± 0.19	-	-	-	-	-
Propanoic acid	1504	-	-	-	-	-	-	0.09 ± 0.14	-	-
3-Methylvaleric acid	1761	-	-	0.06 ± 0.11	-	-	-	-	-	-
Isovaleric acid	1781	-	-	-	-	-	-	0.1 ± 0.14	-	-
Ingol 12-acetate	2908	-	-	-	-	-	-	-	-	-
Alcohol										
n-Butanol	1071	0.68 ± 0.22	3.11 ± 1.38	-	8.21 ± 8.22	0.11 ± 0.1	-	-	-	-
Isoamyl alcohol	1152	-	-	2.69 ± 3.09	-	-	-	-	-	-

1-Butanol, 2-methyl-	1199	0.11 ± 0.1	0.6 ± 0.26	1.43 ± 2.15	2.48 ± 1.55	0.23 ± 0.29	-	-	-	-
Prenol	1242	-	0.08 ± 0.04	-	-	-	-	-	-	-
Isohexanol	1259	-	0.04 ± 0.07	-	-	-	-	-	-	-
(Z)-Hex-3-en-1-ol	1312	-	-	-	0.19 ± 0.14	-	1.87 ± 1.44	-	-	-
Hexan-1-ol	1320	42.87 ± 10.75	88.65 ± 4.15	75.26 ± 40.45	80.59 ± 5.62	98.05 ± 2.53	-	0.44 ± 0.37	-	-
5-Hepten-2-one, 6-methyl-	1398	-	-	-	-	-	-	0.94 ± 0.42	-	-
3-Hexen-1-ol	1415.	12.28 ± 21.2	0.57 ± 0.21	-	-	-	-	0.23 ± 0.1	-	-
n-Heptan-1-ol	1416	0.21 ± 0.02	0.53 ± 0.33	0.91 ± 1.48	0.44 ± 0.3	0.21 ± 0.28	-	0.12 ± 0.04	-	-
1-Octen-3-ol	1429	0.01 ± 0.01	0.18 ± 0.09	-	0.04 ± 0.03	-	-	-	-	-
Ethanol, 2-butoxy-	1432	0.01 ± 0.01	-	-	0.22 ± 0.16	-	-	-	-	-
6-Methyl-5-hepten-2-ol	1436	0.03 ± 0.01	-	-	-	-	0.15 ± 0.1	-	-	-
(E)-Hex-2-en-1-ol	1486	-	0.14 ± 0.09	-	-	-	-	-	-	-
Octan-1-ol	1510	3.71 ± 1.34	2.86 ± 2.82	10.13 ± 17.55	0.73 ± 0.67	0.97 ± 1.31	-	0.14 ± 0.04	-	-
1-Nonanol	1608	-	0.02 ± 0.02	0.01 ± 0.01	-	-	-	-	-	-
3-Nonen-1-ol	1609	-	0.02 ± 0.02	0.13 ± 0.22	-	-	-	0.02 ± 0.03	-	-
2-Octen-1-ol, (Z)-	1679	-	-	-	0.03 ± 0.03	-	-	-	-	-
Hexadecan-1-ol	1935	-	-	-	-	-	0.26 ± 0.22	-	-	-
2-Propen-1-ol, 3-phenyl-, formate	2094	0.01 ± 0.01	-	-	-	-	-	-	-	-
2-Propen-1-ol, 3-phenyl-	2238	0.74 ± 0.96	-	-	-	-	-	-	-	-
Hexadecen-1-ol, trans-9-	2413	-	-	-	-	-	0.05 ± 0.05	0.29 ± 0.44	-	-
Aldehyde										
1,5-Hexanediol	1915	0.03 ± 0.02	0.1 ± 0.12	0.09 ± 0.16	0.24 ± 0.18	0.04 ± 0.05	-	-	-	
Octanal	1295	-	0.06 ± 0.12	-	0.25 ± 0.3	-	-	0.58 ± 0.16	-	-

2-Octenal, (E)-	1401	0.01 ± 0.01	0.13 ± 0.18	0.12 ± 0.21	-	0.02 ± 0.02	-	-	-	-
Nonanal	1472	-	0.18 ± 0.15	0.48 ± 0.83	0.32 ± 0.26	0.05 ± 0.07	-	0.74 ± 0.25	-	-
Phenylacetaldehyde	1622	-	-	-	-	-	0.25 ± 0.2	0.57 ± 0.3	-	0.4 ± 0.14
5-Octen-1-ol, (Z)-	1627	-	-	0.36 ± 0.62	-	-	-	-	-	-
1,6-Hexanediol	2131	0.01 ± 0.01	0.01 ± 0.02	-	0.03 ± 0.03	0.01 ± 0.01	-	-	-	-
Ester										
Phenylethyl acetate	1904	-	-	-	-	-	0.46 ± 0.13	0.31 ± 0.11	0.12 ± 0.11	0.06 ± 0.01
Acetic acid, cinnamyl ester	1919	0.01 ± 0.01	-	-	-	-	-	-	-	-
Hexyl Acetate	1269	0.44 ± 0.27	0.71 ± 0.54	4.77 ± 7.56	4.13 ± 3.15	0.06 ± 0.11	-	-	-	-
Butanoic acid, hexyl ester	1448	-	0.12 ± 0.03	0.23 ± 0.4	0.2 ± 0.27	0.03 ± 0.03	-	-	-	-
Acetic acid, phenylmethyl ester	1729	0.08 ± 0.06	0.01 ± 0.004	-	-	-	-	-	-	-
Benzyl formate	1744	0.05 ± 0.01	-	-	-	-	-	-	-	-
2-Phenylethyl formate	1900	-	-	-	-	-	-	0.28 ± 0.21	0.21 ± 0.05	0.12 ± 0.02
Elemicin	1928	-	-	-	-	-	0.29 ± 0.36	0.27 ± 0.17	-	-
Glycol										
2,3-Butanediol	1514	-	0.01 ± 0.02	-	-	-	-	0.04 ± 0.07	-	-
Aromatic										
Alcohol										
Benzyl alcohol	1908	37.86 ± 15.36	0.38 ± 0.2	0.3 ± 0.5	0.53 ± 0.43	0.06 ± 0.07	-	0.47 ± 0.13	0.22 ± 0.22	-
Phenylethyl alcohol	1910	0.06 ± 0.02	0.12 ± 0.06	2.72 ± 5.05	0.48 ± 0.43	-	87.56 ± 5.57	91.74 ± 1.91	88.71 ± 10.06	94.26 ± 6.46
benzenoids & Phenylpropanoids										
Benzene, 1,4-dichloro-	1469	-	-	-	0.14 ± 0.11	-	-	0.42 ± 0.27	-	-
Benzaldehyde	1557	-	-	-	-	-	-	0.35 ± 0.21	-	-
3,4-Dimethoxytoluene	1901	-	-	-	-	-	0.04 ± 0.05	0.04 ± 0.05	-	-
Creosol	1913	-	-	-	-	-	0.59 ± 0.45	-	0.07 ± 0.1	-

p-Cresol	1920	0.04 ± 0.01	0.04 ± 0.03	0.22 ± 0.43	0.05 ± 0.04	-	1.01 ± 1.16	0.2 ± 0.06	-	-
Eugenol	2165	-	-	-	-	-	7.28 ± 4.03	1.33 ± 1.45	10.66 ± 10.34	5.14 ± 6.57
Other										
m/z: 204*,93,69,41,91,133	1618	0.01 ± 0.01	-	-	-	-	-	-	-	-
Terpenes										
monoterpenes										
Linalool	1522	0.02 ± 0.02	0.02 ± 0.03	-	-	-	-	-	-	-
Terpinen-4-ol	1631	0.02 ± 0.02	-	-	-	-	-	-	-	-
sesquiterpenes										
Alloaromadendrene	1642	0.01 ± 0.01	-	-	-	-	-	-	-	-
Carvone	1750	-	-	-	-	-	0.08 ± 0.07	-	-	-

[1]Compounds which could not be identified with certainty are presented as ***Other***, with the six largest mass fragments in descending order indicated.