Evolution of the *Satyrium longicauda* (Orchidaceae) species complex

by

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PREFACE

The work reported in this thesis was carried out in the Republic of South Africa at the School of Life Sciences, University of KwaZulu-Natal, Pietermaritzburg campus, under the supervision of Dr. Timotheüs van der Niet and Prof. Steven D. Johnson.

These studies represent original work by the author and have not otherwise been submitted in any form for any degree or diploma to any tertiary institution. Where use has been made of the work of others it is duly acknowledged in the text.

Miguel Castañeda Zárate

10 November 2021

I certify that the above statement is correct

Dr. Timotheüs van der Niet (supervisor)

As the candidate's supervisor I have approved this thesis for submission

Dr. Timotheus van der Niet (supervisor)

Prof. Steven D. Johnson (co-supervisor)

DECLARATION 1 - PLAGIARISM

I, Miguel Castañeda Zárate, declare that

1. The research reported in this thesis, except where otherwise indicated, is my original research.

2. This thesis has not been submitted for any degree or examination at any other university.

3. 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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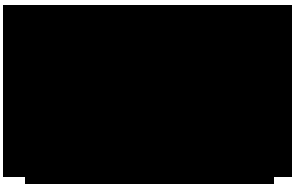
DECLARATION 2 - PUBLICATIONS

The second Chapter entitled "Food Reward Chemistry Explains a Novel Pollinator Shift and Vestigialization of Long Floral Spurs in an Orchid" has been published in *Current Biology* 31 (1): 238-246.e7.



Miguel Castañeda Zárate

10 November 2021

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Dr. Timotheus van der Niet (supervisor)

Prof. Steven D. Johnson (co-supervisor)

ABSTRACT

The identification of morphological variation in taxa grouped within a species complex is fundamental not only in systematics, but plays an important role in evolutionary biology as well. Finding diagnostic characters among populations in plant species undergoing speciation may be challenging due to the continuous nature and intraspecific overlap of variation in reproductive and vegetative traits. The orchid *Satyrium longicauda* currently comprises two varieties (*jacottetianum* and *longicauda*) which can be identified mainly by differences in spur length. Additionaly, *S. buchananii* and *S. rhodanthum* were proposed as part of the group which represents a species complex. The large variation in phenotype in the species detected across its broad range makes this complex a promising candidate for studying its systematics and evolutionary origin, through the implementation of multi-disciplinary tools.

In this thesis, I have characterised the morphological variation of sympatric and allopatric natural populations of *S. longicauda* by traditional morphometrics and uni- and multivariate analyses leading to the identification of eight morphotypes, including *S. rhodanthum,* that partially overlap in traits. These taxa, together with *S. buchananii* which was not studied in detail, served as units of comparison in a molecular phylogeny that supported the monophyly of some of the morphotypes, but at the same time revealed the non-monophyly of *S. longicauda*. Extensive pollinator data from direct observations and motion-trigger cameras, revealed that most morphotypes are pollinated by nocturnal moths but pollination by long-tongued flies, sunbirds, and oil-collecting bees was also recorded. Analyses of pollinator assemblages using network tools led to identification of five modules representing potential pollination niches. Several evaluated floral traits such as colour, scent chemistry, nectar volume and concentration, and functional spur length co-varied with some or all of the pollination niches, suggesting they could represent functional traits. Traits associated with the sunbird pollination niche were the most divergent compared to traits of the moth, oil-collecting bee, and a mixed pollination niche. Subtle differences in nectar properties and functional spur length could be linked to the oil-collecting bee pollination niche and two nocturnal moth pollination niches, comprising pollination by settling moths and hawkmoths, respectively. A pollinator shift from nocturnal moth to oil-collecting bee pollination was demonstrated using a combination of field-based experiments and a phylogenetic analysis. This shift appears to be

triggered by changes in the type of floral reward from nectar to oil. Morphological variation associated with genetic differences in combination with the identification of an exclusive pollination niche support the recognition of a new variety that is distinct from the two accepted varieties within the complex.

Results presented in this thesis contribute new evidence on pollinator-driven evolution and systematics of *S. longicauda*. At the same time, the thesis provides a starting point for further studies of the processes involved in the diversification of species complexes and for testing the role of pollinators in shaping and maintaining such diversity.

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CHAPTER 1

GENERAL INTRODUCTION

No one knows exactly how many species exist on Earth. One of the latest estimates suggests that the number of species, including yet-to-be-discovered taxa, might be approximately 8.7 million (Mora *et al.* 2011). Plants are relatively well-studied, and a count of accepted published species indicates that ca 348,000-374,000 species inhabit our planet (Christenhusz and Byng 2016). This large species richness can be explained as the result of different factors including environmental stressors (Strauss and Whittall 2006; Jansson and Davies 2008; Shaw *et al.* 2010; Silva *et al.* 2017; Rundel *et al.* 2018), biogeographic history (Moore and Donoghue 2009; Nicolas and Plunkett 2014), and the evolution of novel morphological traits (Hodges and Arnold 1995; Hodges 1997; Gianoli 2004; Silvestro *et al.* 2013). Angiosperms, or flowering plants, comprising about 300,000 species represent by far the most diverse clade of land plants (Hernández-Hernández and Wiens 2020). Their rapid diversification, which is characterised by an astonishing variety of floral forms (Moyroud and Glover 2017) and large species richness compared to other plant clades, has primarily been explained as the result of biotic interactions such as herbivory (Ramos and Schiestl 2019) and especially pollination (Hernández-Hernández and Wiens 2020).

Importance of studying morphological variation

Before trying to identify the likely drivers and mechanisms that explain how extant (and extinct) diversity originated, it is essential to characterise and quantify morphological variation of different organisms. Owing to the fact that morphological characters often give insight into whether two similar species differ from each other, it is reasonable to first evaluate the presence of morphological discontinuities. Throughout history, humans have instinctively classified organisms based on perceived visual similarities and differences. The first example of classification dates back to Aristotle (384–322 BC), who relied heavily on direct observation to classify all living things according to their physical similarities (Pratt 1982; Manktelow 2010). For instance, he separated plants into trees, shrubs, and herbs, whereas animals where categorised into two groups, blooded and bloodless animals (Lloyd 1961). With the advent of taxonomy as a discipline that identifies, describes, and classifies species (Raven *et al.* 1971; Padial *et al.* 2010; Schlick-Steiner *et al.* 2010; Lücking 2020), Aristotle´s system was replaced with the Linnean binomial system (that introduced standard hierarchies) based on the combination of two Latin names for genus and species (Paterlini 2007). This system is still used today.

The presence of natural variation observed in biological systems represents an important study subject in different fields within the broad area of biological sciences. Patterns of morphological variation among and within individuals of the same species can be used for the recognition of hitherto unknown species or taxa below the species level. The most straightforward method for quantifying variation at the specific and infraspecific taxonomic levels requires detailed morphological studies, that could be based on morphometrics and univariate and multivariate statistical analyses (Henderson 2006; Mutanen and Pretorius 2007; Marhold 2011). Widespread species often exhibit intraspecific morphological variation as a consequence of adaptation to a range of environmental conditions. If variation that is represented in multiple populations suggests the presence of more than one species, but phenotypic similarities or insufficient data render species boundaries unclear, and if populations are closely related, it is likely that this represents a species complex (Brown *et al.* 1995; Sigovini *et al.* 2016; Pinheiro *et al.* 2018). Thus, species complexes constitute groups of potentially incipient species in which diversification is an ongoing process, and may be associated with low levels of genetic differentiation. Moreover, species complexes sometimes comprise not only varieties or subspecies of one taxon but also morphologically well differentiated species (Duminil *et al.* 2012; Moroni *et al.* 2016; Pinzón *et al.* 2016) that may represent progenitor-derivative species which have adapted to different habitats and arise from peripheral populations (Crawford and Smith 1982; Crawford 2010; Schlüter *et al.* 2011; López *et al.* 2012). Therefore, members of species complexes are not only morphologically similar but also phylogenetically related (Johnson and Linder 1995; Adhikari and Wallace 2014; van der Niet, Pirie, *et al.* 2014; Pessoa *et al.* 2021). The taxonomic rank given to members of a species complex (cf. Grant and Grant 1965) depends on the taxonomist knowledge and preferences, but they are often placed in the subspecies or variety category (Hamilton and Reichard 1992). Although species complexes pose challenges to the taxonomist, they provide opportunities for studying evolution as on ongoing process. This is because intraspecific variation represents early stages of divergence and understanding the patterns and processes associated with the evolution of intraspecific divergence may hold the key to understanding what drives morphological divergence and speciation. These processes are much more difficult to study above the species level, where both extinction and a long evolutionary time since divergence may obscure drivers of diversification.

The study of intraspecific variation was historically performed by naturalists and biosystematists when museum taxonomy was primarily monographic. Darwin identified variation below the species level (varieties, subspecies) as important components of the formation of species (Mallet 2007; Winker 2010). Moreover, intraspecific variation was important in the formulation of his theory of evolution (Mallet 2007; Winker 2010; Bolnick *et al.* 2011). Although clearly important, studies of intraspecific variation by both systematists and ecologists have reduced in number after 1970s (Stace 1981; Bolnick *et al.* 2011). Nowadays, most comparative studies focus on characterising patterns of morphological and genetic variation at the species level or higher taxonomic ranks (Butterworth and Wallace 2004; Ponsie *et al.* 2007; Pessoa *et al.* 2012; Costa *et al.* 2015). This tendency may represent a setback to the understanding of natural systems, as evolutionary studies often require information that needs to be finely documented through observation of organisms in populations in their natural environments (Ogilvie 2003; van der Niet 2021).

The concept of intraspecific variants of a species that are adapted to particular environmental conditions or habitats, and that are not only morphologically but also genetically distinct, is referred to as ecotypes (Turesson 1922a). This concept was coined by Turesson (1922a) based on his identification of repeated patterns of intraspecific variation of coastal and inland populations of about twenty plant species across their extensive natural distribution. It has since served an important purpose in the study of the constitution and origin of species (Turrill 1946). Clausen (1951) suggested that ecotypes constitute incipient species, as they are not only spatially but also reproductively isolated by ecological barriers including temporal flowering isolation and pollinator isolation. Over the past century, a series of studies has tried to experimentally determine whether phenotypic differences among populations are due to genetic or environmental factors. Reciprocal transplant experiments are used to detect local adaptation by measuring fitness components, and/or common gardens experiments are used to determine whether intraspecific variation arises from plasticity or has a genetic basis (Turesson 1922a; b; Clausen 1951; McMillan 1959; Galen *et al.* 1991; Rice and Mack 1991; Anderson *et al.* 1996, 2021; Bender *et al.* 2002; Angert and Schemske 2005; Lowry *et al.* 2008). These studies revealed that phenotypic divergence among allopatric populations is often genetically based and that individuals of local ecotypes outperform individuals of foreign ecotypes in their respective habitats. Depending on whether ecotypes are reproductively isolated, taxa within a single species that are found to be morphologically and

genetically discrete can eventually be described as different species (Johnson and Linder 1995; Johnson and Steiner 1997; Peter and Johnson 2014).

Modern taxonomy integrates data derived from different fields such as anatomy, chemistry, ecology, cytologenetics, and phylogenetics, and uses evidence from different types of data including morphology, DNA sequencing, karyology, and pollination biology, for the recognition of undescribed species, a practice often referred to as integrative taxonomy (Etota *et al.* 2010; Schlick-Steiner *et al.* 2010; Jakubska-Busse *et al.* 2012; Slovák *et al.* 2012; Vela Díaz 2013; Spooner 2016). An integrative taxonomy has the potential to contribute to a more objective way of quantifying variation at different levels and will add important evidence for delimiting and classifying species. Furthermore, the implementation of integrative taxonomy may provide more objectivity in the important task of delimitating diversity. Moving from a merely descriptive traditional taxonomy to a more integrative (modern) taxonomy that not only identifies, describes and classifies species but also simultaneously tries to understand their origin, establish their limits and reconstructs the evolution of species (Dayrat 2005; Will *et al.* 2005; Padial *et al.* 2010; Schlick-Steiner *et al.* 2010; Pante and Schoelinck 2015) may allow for the identification and studying of the potential abiotic (e.g. drought, UV-B irradiance, soil properties), and biotic (e.g. pathogens, herbivores, pollinators) drivers of plant diversification.

Pollinators as drivers of angiosperms diversity

One major aspect of the diversity of flowering plants includes the presence of specific patterns of floral similarity among distantly related species that share pollinators. Floral traits such as shape, orientation, colour, scent, and reward in specific combinations can be associated with pollination by particular functional animal groups. Suites of such floral traits that represent convergent adaptations of plants to particular functional pollinator groups, that is, pollination syndromes, have been used to organise floral diversity under a functional-ecological perspective (Faegri and van der Pijl 1979; Vogel 2012; Dellinger 2020). Pollination syndromes have often been used to reliably predict pollinators in the absence of empirical observations (Martén-Rodríguez *et al.* 2009; Armbruster *et al.* 2011; Rosas-Guerrero *et al.* 2014; Johnson and Wester 2017). For instance, traits that characterise plants pollinated by nocturnal moths include pale, green or white flowers that release a strong sweet scent at night and offer nectar in long tubular corollas or spurs (Faegri and

van der Pijl 1979; Vogel 2012). However, pollination syndromes are not always indicative of the correct functional pollinator group, which is why the concept has received mixed support (Waser 2006; Martén-Rodríguez *et al.* 2009; Ollerton *et al.* 2009; Xie *et al.* 2013; Johnson and Wester 2017; Wang *et al.* 2020).

If a plant population adapts to a novel pollinator, a pollinator shift (transition between functional pollinator groups) has occurred. These pollinator shifts are associated with modification of floral traits that allow utilisation of different pollinators (Stebbins 1970; Hodgins and Barrett 2008; Roalson and Roberts 2016). Given that pollinators mediate plant reproduction, pollinator shifts often result in divergence in floral traits in combination with assortative mating, leading to the evolution of prezygotic reproductive isolation, i.e. the formation of biological species (Grant 1994; Ramsey *et al.* 2003; Christianini *et al.* 2013; Sobel and Streisfeld 2015). Hence, shifts between pollinators are considered important drivers of plant speciation and diversification (van der Niet and Johnson 2012). A combination of species-level phylogenetics and pollinator data can provide important information regarding patterns of pollinator shifts in terms of their frequency, direction, and floral modifications associated with the shift. For instance, the pollination syndromes of species in the African genus *Clivia* suggested the presence of pollination by birds and butterflies respectively. Indeed, using phylogenetic analyses, modifications in flower shape and orientation, reduction in the production of nectar, and the increase in the number of floral scent compounds and scent emission were found to be associated with a pollination system transition from sunbird- to butterfly pollination (Kiepiel and Johnson 2014).

Although macroevolutionary analyses provide insight into patterns of pollinator-driven diversification, they do not provide insight into the process. This requires a population-level approach. The way in which plants attract pollinators is by using a wide variety of visual and olfactory floral signals such as shape, colour, and scent (Willmer 2011). Animals lured by these advertisements pick up and deposit pollen on the stigmas of other plants while usually seeking nutritive rewards (Simpson and Neff 1981). Given the presence of specific sensory preferences of pollinators, they act as selective agents of floral traits (Goyret *et al.* 2008; Yoshida *et al.* 2015). According to the Grant–Stebbins model of pollinator-driven divergence (Grant and Grant 1965; Stebbins 1970; Johnson 2006, 2010), pollinators are considered the agents of divergent selection on floral traits by promoting plants to locally adapt to the most frequent and effective pollinators.

In this conceptual model, pollinators interact with the floral traits depending on their sensory preferences in relation to floral advertisement traits, and with floral morphology in terms of the removal and deposition of conspecific pollen (pollinator effectiveness). Hence, pollinators represent an important selective force for the evolution of visual and olfactory floral traits such as shape, colour, and scent (Stebbins 1970; Faegri and van der Pijl 1979; Alexandersson and Johnson 2002; Gegear and Burns 2007; Whittall and Hodges 2007; Schiestl and Johnson 2013; Shrestha *et al.* 2013; Fenster *et al.* 2015).

The link between the divergence of floral traits that can be observed and quantified, and the evolution of reproductive isolation, provides a critical bridge between the Biological and Taxonomic Species Concept, and allows taxonomists to recognise the products of pollinator-driven divergence as separate taxonomic units that may form the basis for describing new taxa (e.g. Johnson and Linder 1995; Johnson and Steiner 1997). An important point to consider is that the selection of a species concept is an important step in the task of naming a new species. In the context of pollinator-driven reproductive isolation, the Biological Species Concept represents the most influential species concept for defining species boundaries (Mayr 1942). Characters used in taxonomy for species delimitation should ideally be based on discrete variation that can be unambiguously identified in type specimens. However, incipient species or recently diverged species that have undergone a pollinator shift, may lack non-overlapping diagnostic characters. Depending on the dimensions of the variation and the tools used to quantify it, taxonomists can determine whether the observed variation is sufficiently discrete to draw species boundaries. Otherwise, the description of new species that are difficult to identify due to a lack of diagnostic morphological (and genetic) characters may result in taxonomic inflation (Padial and De La Riva 2006; Zachos *et al.* 2013).

Due to the fact that not all pollinators are equally effective (Stebbins 1970), and exhibit an uneven distribution in space and time and vary in their abundance, both pollinator efficiency and geographical differences in pollinator assemblages can promote population-level floral adaptive divergence across plant species ranges (Grant and Grant 1965). The process of floral phenotypic divergence can thereby lead to the evolution of pollination ecotypes (Grant and Grant 1965). Supported by ecological and phylogenetic evidence, the model of pollinator-driven diversification according to which groups of floral traits are shaped by particular functional groups of pollinators

is often invoked to explain plant diversification through the formation of pollination ecotypes (Smith 2010; van der Niet and Johnson 2012; van der Niet, Peakall, *et al.* 2014). Empirical support for the role of pollinators in divergence of floral traits has been provided by studies that documented associations between variation in floral traits and pollination systems, and sometimes included the implementation of reciprocal transplant experiments in allopatric populations (Table 1.1). These studies show how pollinators drive phenotypic variation in both generalist and specialist plant species. Eventually, floral traits selected to attract different pollinators may result in the evolution of floral isolation that keep species reproductively isolated (Grant 1949; Johnson and Linder 1995; Johnson 1997a, 2006; Kay and Sargent 2009; Xu *et al.* 2012; Whitehead and Peakall 2014).

Although substantial work has been done in trying to understand the role of pollinators in plant diversification, most studies focus on evidence for transitions between major pollination systems (e.g. insect vs bird pollination). However, divergence in morphological floral traits and mechanical fit between pollinators belonging to the same major pollinator group (e.g. different species of moths) has been less studied (but see Anderson *et al.* 2010; Boberg *et al.* 2014). In addition, a phylogenetic component has been seldom implemented in studies of pollinator-driven divergence at the species level. Knowledge of phylogenetic relationships among members of a species complex not only is useful for identifying the presence and direction of pollinator shifts, but can also provide evidence that can be used in taxonomy for the recognition of different taxa (varieties, subspecies or even species). Another gap in knowledge arises from the fact that most pollination ecotypes occur, and are studied in an allopatric context. Studies of natural populations that show an overall similar phenotype and continuous variation in floral traits, and that occur in sympatry rather than allopatry, are rare. Hence, what remains unclear is what drives divergence in floral traits without major shifts in phenotype. An integrative study of species complexes that exhibit subtle variation in traits that indicate pollination by species within the same pollinator group, and where many morphological variants coexist and co-flower, may help to understand the mechanisms involved in evolutionary divergence and speciation in plants.

Table 1.1. Selected pollination ecotype studies performed in the last four decades. Evaluated traits that support the recognition of pollination ecotypes are shown in bold.

Plant species and geography	Intraspecific units	Reward	Functional pollinator group	Evaluated traits	Methods	Reference (s)
Dalechampia scandens (Euphorbiaceae) Mexico, Belize, Guatemala, Costa Rica, Panama, Ecuador, Brazil	—	resin	various resin-collecting bee species within genera *Hypanthidium*, *Euglossa*, *Trigona*, and *Eulaema*	**resin-gland area**, **gland-stigma distance**, **gland-anther distance**, **anther-stigma distance**, time of opening of inflorescences	flower-pollinator trait associations, pollinator observations, common garden experiments	Armbruster 1985
Platanthera ciliaris (Orchidaceae) United States	mountain	nectar	butterfly (*Papilio troilus*)	**lateral sepal width**, distance between viscidia, **stigma width**, **nectary- orifice diameter**, **spur length**, labellum length, **lateral fringe length**, **number of flowers per plant**, **number of fruits matured**, **percentage fruit-set per plant**	flower-pollinator trait associations, pollinator observations, reciprocal transplants	Robertson and Wyatt 1990
	coastal plain		butterfly (*Papilio palamedes*)			

	Disa harveiana subsp. *harveiana* (southern mountain)		horsefly (*Philoliche rostrata*)			
Disa draconis (Orchidaceae) South Africa	*Disa draconis* (sandplain)	food deception	tanglewing fly (*Moegistorynchus longirostris*)	flower colour, **flowering time** and eight morphological floral traits including **spur length**, dorsal sepal length, lateral sepal length, petal length	flower-pollinator trait associations, pollinator observations, manipulative experiments	Johnson and Linder 1995; Johnson and Steiner 1997
	Disa harveiana subsp. *longicalcarata* (northern mountain)		horsefly (*Philoliche rostrata*?)			
	subsp. *hallackii* (coastal fynbos)		bees (*Xylocopa caffra*, *X. capitata*, *Allopade panurgoides*)			
Satyrium hallackii (Orchidaceae) South Africa	subsp. *ocellatum* (grassland)	nectar	hawkmoths (*Basiothia schenki*, *Hippotion celerio*, *Agrius convolvuli*) and long-tongued flies (*Prosoeca ganglebauri*)	**spur length**, **nectar volume**, **nectar concentration**, sugar composition	flower-pollinator trait associations, pollinator observations	Johnson 1997

Gladiolus longicollis (Iridaceae) South Africa	short-tubed long-tubed	nectar	short-tongued hawkmoth (*Basiothia schenki*) long-tongued hawkmoth (*Agrius convolvulli*)	inflorescence height, flower diameter, **corolla tube length**, **floral scent**, nectar volume, **nectar concentration**	flower-pollinator trait associations, pollinator observations	Alexandersson and Johnson 2002; Anderson *et al.* 2010
Disa spathulata (Orchidaceae) South Africa	subsp. *spathulata* (western part of the Cape region) subsp. *tripartite* (southern part of the Cape region)	food deception	bee (*Tetraloniella brevikeraia*) bee (*Tetraloniella junodi*)	**floral scent**	flower-pollinator trait associations, pollinator observations, reciprocal translocation experiments	Johnson *et al.* 2005
Erysimum mediohispanicum (Brassicaceae) Spain	—	nectar and pollen	several hundreds of insects including solitary bees, long-tongued beeflies, short-tongued flies, beetles, and butterflies	stalk height, **flower number**, **corolla diameter**, corolla tube length, **corolla shape**	pollinator observations, pollination niches identification using network tools, flower-pollinator trait associations by traditional and geometric morphometrics, phylogenetic analyses	Gómez *et al.* 2008, 2009, 2014

Disa ferruginea (Orchidaceae) South Africa	orange morph red morph	food deception	butterfly (*Aeropetes tulbaghia*) butterfly (*Aeropetes tulbaghia*)	**flower colour**	flower-pollinator trait associations, pollinator observations, reciprocal translocation experiments	Newman *et al.* 2012
Platanthera bifolia (Orchidaceae) Norway, Sweden	grassland woodland	nectar	short-proboscis hawkmoth (*Deilephila porcellus*) long-proboscis hawkmoth (*Sphinx ligustri*)	**plant height**, **flower length**, **flower width**, **spur length**, **stem height**, number of flowers	flower-pollinator trait associations, pollinator observations, reciprocal translocation experiments, manipulative experiments	Boberg and Ågren 2009; Boberg *et al.* 2014; Trunschke *et al.* 2019
Calceolaria polyrhiza (Calceolariaceae) Chile, Argentina	Andean–Patagonian forests Patagonian steppe plain	oil	oil-collecting bee (*Chalepogenus caeruleus*) oil-collecting bee (*Centris cineraria*)	**style length**, **filament length**, theca length, **throat length**, elaiophore width, corolla area, total leaf length, leaf lamina width, leaf lamina length	flower-pollinator trait associations, pollinator observations	Cosacov *et al.* 2014
Eulophia parviflora (Orchidaceae) South Africa	low altitude (short-spurred) high altitude (long-spurred)	food deception	beetle (*Cyrtothyrea marginalis*) bee (*Amegilla fallax*)	**flowering phenology**, flower colour, **floral scent**, and over sixty traits including **spur length**	flower-pollinator trait associations, pollinator observations, choice experiments, reciprocal translocation experiments	Peter and Johnson 2014

Gymnadenia odoratissima (Orchidaceae) Switzerland	lowland	nectar	butterflies, moths and beetles	**floral scent** **flower colour** **nectar volume, nectar concentration**, and eleven morphological floral traits including inflorescence length**,** **flower width**, **labellum width**, labellum height, **side-lobe length**, interlobe distance, spur length**,** **flower area**	flower-pollinator trait associations, pollinator observations, reciprocal translocation experiments	Sun *et al.* 2014
	mountain		butterflies, moths, flies, and beetles			
Erica plukenetii (Ericaceae) South Africa	subsp. *breviflora*	nectar	noctuid (*Helicoverpa armigera*) and sunbird (*Anthobaphes violacea*)	**synflorescene length**, **corolla length**, pistil length, **nectar volume**, **nectar concentration**, **floral scent**, flower colour	flower-pollinator trait associations, pollinator observations, phylogenetic analyses	van der Niet *et al.* 2014
	subsp. *plukenetii*		sunbird (*Nectarinia famosa*)			

Drakaea concolor (Orchidaceae) Australia	—	sexual deception	thynnine wasp (*Zaspilothynnus gilesi*)	**floral scent**	flower-pollinator trait associations, pollinator observations, choice experiments	Phillips *et al.* 2015
	—		thynnine wasps (*Zaspilothynnus gilesi* and *Pogonothynnus* sp.)			
Nerine humilis (Amaryllidaceae) South Africa	short style	nectar	honey bee (*Apis mellifera*)	**functional style length**, tepal length, **nectar volume**, nectar concentration, **flower colour**	flower-pollinator trait associations, pollinator observations, reciprocal translocation experiments	Newman *et al.* 2015
	long style		long-proboscid flies (*Prosoeca ganglebauri* and *P. longipennis*)			
Claytonia virginica (Portulacaceae) Unites States	northern	nectar and pollen	oligolectic bee (*Andrena erigeniae*)	**pollen depletion**, **pollen presentation**, **total pollen production**	flower-pollinator trait associations, pollinator observations	Parker *et al.* 2016, 2017
	southern		bee fly (*Bombylius major*)			

Overview of the genus *Satyrium*

With approximately 25,000—28,000 species, grouped in five different subfamilies, Orchidaceae is one of the largest families of flowering plants (Chase *et al.* 2015; Christenhusz and Byng 2016). The orchids comprise approximately 8% of the total of described angiosperms. They have a cosmopolitan distribution occurring on all continents except Antarctica (Fay and Chase 2009). The high diversity in the family is thought to be the result of the evolution of various key innovations including the packaging of pollen grains into pollinia, the epiphytic habit, CAM photosynthesis, their tropical distribution, and interactions with animal pollinators (Johnson and Edwards 2000; Gravendeel *et al.* 2004; Givnish *et al.* 2015, and references therein). The subfamily Orchidoideae, with over 3600 species that are mostly terrestrial, is formed by four tribes (Chase *et al.* 2015). Within Orchideae, the subtribe Orchidinae with about 60 genera is the most diverse, comprising more than 1800 species (Chase *et al.* 2015; Jin *et al.* 2017) .

Satyrium Sw. (Orchidinae, Orchidoideae) comprises about 100 species mostly distributed in Africa, with only 4 species ranging into the Himalayan region of Asia (Hall 1982; Kurzweil and Linder 2001; van der Niet *et al.* 2005; Johnson *et al.* 2011). The genus has centres of diversity in the grasslands of southern and south-central Africa, and in the fynbos shrublands of the south-western Cape in South Africa (van der Niet *et al.* 2005; van der Niet and Johnson 2009). Members of this genus can be recognised by their non-resupinate flowers with a hooded labellum that includes two spurs (Figure 1.1; Hall 1982; Linder and Kurzweil 1994; Kurzweil 1996). Taxonomic revisions of the genus have emphasised the extensive variation in both vegetative and floral traits (Hall 1982; Kurzweil and Linder 1999). Vegetative traits such as plant size, leaf shape and orientation, and floral traits such as colour, labellum and rostellum shape and position, and spur length are among the most variable. In taxonomy, the length of the spur has played an important role in the delimitation of species and particularly in the recognition of supraspecific taxa named subspecies or varieties (Hall 1982; van Der Niet and Cribb 2006; Kurzweil *et al.* 2009). Overall, due to the presence of extensive intra- and interspecific variation, several species complexes within the genus have been identified (Hall 1982).

Ecological studies in the genus have revealed that the tremendous variation in morphological traits is matched by variation in flower colour, scent, and rewards that are used to entice floral visitors (Johnson *et al.* 2011; van der Niet, Jürgens, *et al.* 2015). Currently, the pollination systems of over twenty species have been documented. Reports of pollination by carrion flies, anthophorid bees, oil-collecting bees, bumblebees, sunbirds, long-tongued flies, beetles, butterflies, nocturnal moths

(noctuids and hawkmoths), and even autonomous self-pollination make the genus one of the most diverse in terms of pollination systems (Garside 1922; Johnson 1996, 1997b; Harder and Johnson 2005; Jersáková and Johnson 2007; Johnson *et al.* 2007; Huang *et al.* 2009; Ellis and Johnson 2010; Johnson *et al.* 2011; van der Niet, Hansen, *et al.* 2011; Duffy and Johnson 2014; van der Niet *et al.* 2015; van der Niet, Jürgens, *et al.* 2015; van der Niet 2018; Botes *et al.* 2020). The association between floral traits and particular pollination systems implies that pollinators are important for promoting selection of floral traits and diversification in the genus (Johnson *et al.* 2007, 2011; van der Niet, Hansen, *et al.* 2011). For example, the study by Johnson (1997) illustrated intraspecific variation in floral traits in the two subspecies of *Satyrium hallackii* and attributed this to pollination by different pollinators, determined by their respective distributions. Specifically, the variation in spur length and flower colour led to identification of a pink short-spurred ecotype pollinated by carpenter bees and a light pink long-spurred ecotype pollinated by hawkmoths and long-tongued flies.

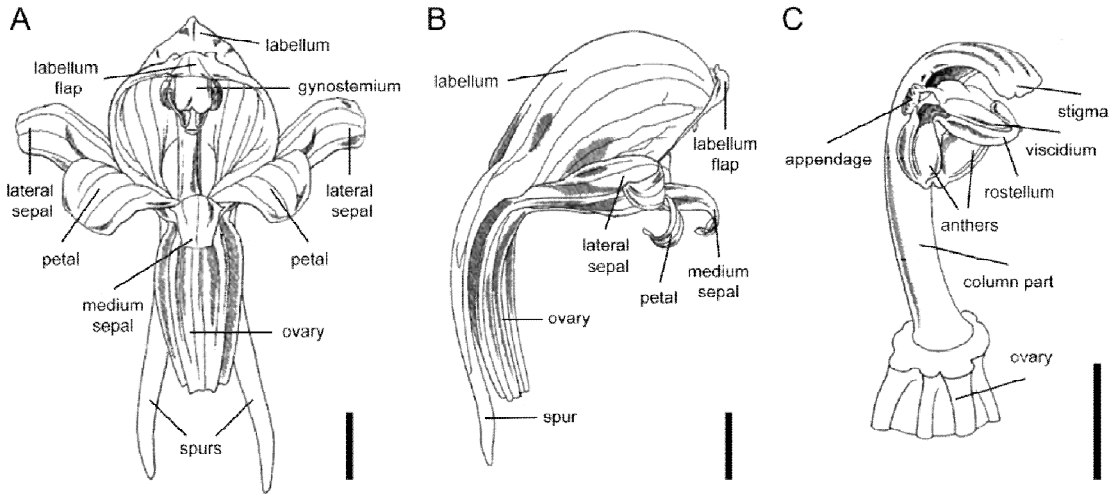

Figure 1.1. Floral morphology of *Satyrium*. A-B) Flower of *S. carneum* in ventral and lateral view. C) Gynostemium. Modified from Kurzweil, 1996. Scale bar = 5 mm.

The *Satyrium longicauda* (Orchidaceae) species complex

Satyrium longicauda Lindl., with scented white to pink flowers with spurs that offer nectar as reward, represents one of the several species complexes identified in the genus (Hall 1982; Johnson *et al.* 2011). Members within this species complex have so far been reported from Eastern and Southern African countries including Tanzania, Malawi, Zambia, Mozambique, Zimbabwe, Eswatini, Lesotho and South Africa (Figure 1.2; Hall 1982). In his revision of the southern African species of

Satyrium, which was mostly based on herbarium specimens, Hall (1982) detected extensive morphological variation in floral and vegetative traits of *S. longicauda*. Furthermore, after observing individuals with divergent morphology co-flowering only a few meters apart, he hypothesised that such intraspecific variation may be the result of single gene mutations. He also suggested that future studies would allow the recognition of different taxa within the Forma taxonomic rank. Although the presence of sympatric populations representing different forms was recognised, the current taxonomy accepts only two varieties within the species. These mostly differ in the size of floral segments, particularly the length of the spur.

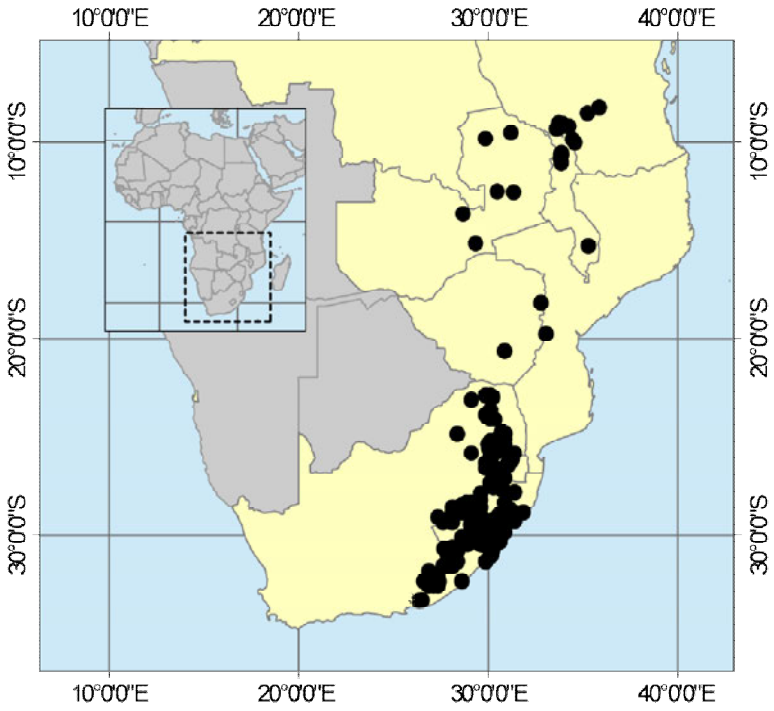

Figure 1.2. Map showing the distribution of the *Satyrium longicauda* complex in Southern Africa.

The short-spurred variety known as var. *jacottetianum* (Kraenzl.) A. V. Hall, initially described as a different species from *S. longicauda*, has its leaves adpressed to the ground, and has reddish flowers with spurs 13-26 mm long, and lateral sepals 4-7 mm long (Hall 1982). It is distributed at elevations chiefly between 1300 and 1900 m. The long-spurred variety, var. *longicauda*, is considered to have one or two leaves that vary in their orientation, white to pink flowers with spurs 24-46 mm long, and lateral sepals 5-11 mm long. It overlaps in distribution with var. *jacottetianum*. Although Hall (1982) did not have access to a large number of specimens of *S. buchananii*, he did find morphological similarities with *S. longicauda*. *Satyrium buchananii* is characterised by white flowers with spurs 44-

75 mm long and lateral sepals 8-12 mm long, can sometimes be found in sympatry with *S. longicauda*, and may represent a potential third variety. Another species, the extremely rare *S. rhodanthum* Schltr., that is endemic to a small region in South Africa, is characterised by unscented red flowers and was initially considered a taxon of hybrid origin between *S. longicauda* and *S. neglectum* ssp. *woodii* (Hall 1982). Currently, it is accepted as a different species from *S. longicauda*, but their affinities have not yet been evaluated (Linder and Kurzweil 1999; Johnson and Bytebier 2015). In recent years, phylogenetic analyses that were used to reconstruct the relationship among *Satyrium* species showed that *S. buchananii* is nested inside *S. longicauda* (van der Niet *et al.* 2005; van der Niet and Linder 2008; van der Niet, Liltved, *et al.* 2011).

Floral traits such as white flowers that emit a sweet fragrance and offer nectar in floral spurs are characteristic of the moth pollination syndrome (Faegri and van der Pijl 1979). Indeed, several studies that included aspects of the reproductive ecology of South African populations of both varieties of *S. longicauda* have confirmed pollination by nocturnal moths, either noctuids or hawkmoths (Harder and Johnson 2005; Jersáková and Johnson 2007; Ellis and Johnson 2010; Johnson *et al.* 2011; Duffy and Johnson 2014). Furthermore, pollination of *S. rhodanthum* by sunbirds has been documented (van der Niet *et al.* 2015). Studies of taxa within the complex outside South Africa are absent; therefore, the pollinators of either of the two varieties of *S. longicauda* and *S. buchananii* that occur in Zimbabwe, Zambia, Malawi, and Tanzania are unknown.

Given the wide distribution of the *S. longicauda* complex, particularly in South Africa where populations range from near to sea level in KwaZulu-Natal and the Eastern Cape up to ~ 3100 m in the eastern portion of the Great Escarpment (specifically the Maloti-Drakensberg mountain system), and the extensive morphological variation in vegetative and floral traits observed in the group, this species is an ideal candidate to study the potential drivers that explain such variation. A first step is to characterise the morphological and genetic variation of sympatric and allopatric populations, in order to identify the units of divergence and determine whether the current taxonomy that recognises two varieties is adequate or in need of updating. In addition to morphometric analyses of floral and vegetative traits, studies of the breeding system and pollination systems with associated characters may reveal whether differences in floral traits are linked to differences in the pollinator fauna, which would lead to recognise pollinators as important drivers of the variation observed in the complex. Traits under investigation are those that are considered important in the pollination syndrome concept such as flower colour, scent, spur length (which often correlates with the proboscis of the pollinators), and the presence and nature of floral rewards. Ultimately, the *Satyrium*

longicauda complex represents an ideal study system that may help to understand how minor changes in floral morphology are associated with the utilisation of pollinators in allopatric and sympatric populations.

Thesis outline

The general aim of this research was to characterise the morphological variation of natural populations of the *S. longicauda* complex (Orchidaceae) and to evaluate the role of pollinators in the evolution of the complex. I hypothesised that morphological variation in vegetative, but particularly subtle differences in floral traits of sympatric and allopatric populations, is associated with different pollination systems. Thus, populations with divergent floral traits are likely the result of adaptation to different pollinators and may represent previously unknown taxa. In addition, the description of the pollination systems in the group would allow a deeper understanding of whether pollinators are relevant in the diversification of the complex.

Chapter 2 describes the discovery of a novel pollination system, based on a study of pollination systems and associated floral traits of six populations occurring in sympatry. Using a phylogenetic framework, I show that a pollinator shift from nocturnal moths to oil-collecting bees occurred as a consequence of changes in floral reward.

Chapter 3 has a much broader geographical scope and evaluates whether combining morphometric and phylogenetic analyses for the study of natural populations of eight distinct morphotypes that were initially identified through visual evaluation, results in the identification of independent lineages. Using this framework, Chapter 4 uses a large dataset of pollinator observations and floral trait quantifications to test whether the plant-pollinator network of *S. longicauda* is modular and whether floral trait variation is associated with discrete pollination niches.

In Chapter 5, I describe and illustrate a new variety within the complex: *S. longicauda* var. *redivivense* from KwaZulu-Natal. The description is the result of combining morphological, genetic and ecological evidence gathered in Chapters 2, 3, and 4.

Finally, Chapter 6 summarises the results obtained in the previous chapters and discusses how the study of intraspecific variation by means of different tools in the context of an integrative taxonomy,

with a particular focus on reproductive biology and systematics, contributes to understanding of the evolution of the species complex.

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CHAPTER 2

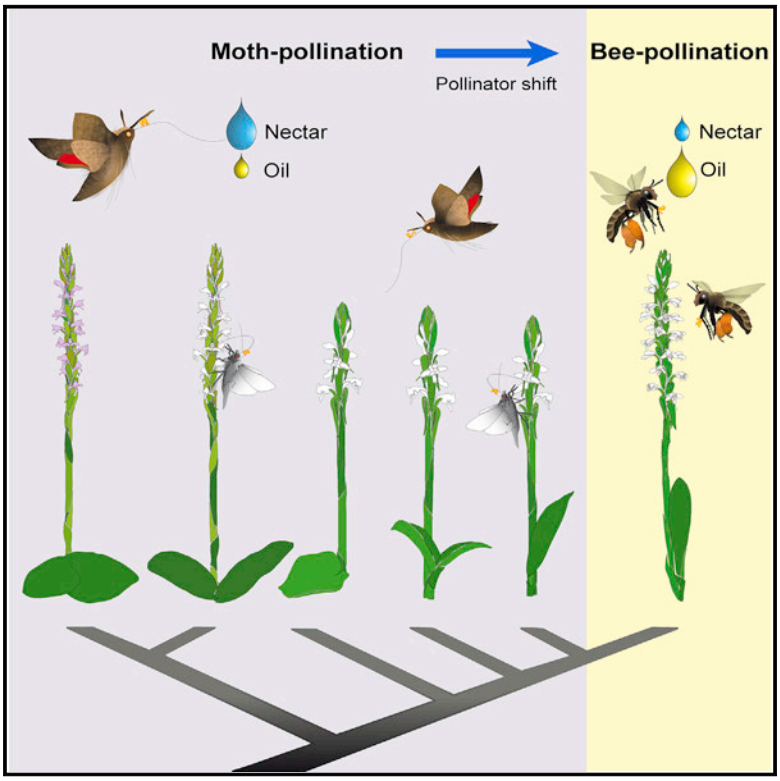
FOOD REWARD CHEMISTRY EXPLAINS A NOVEL POLLINATOR SHIFT AND VESTIGIALIZATION OF LONG FLORAL SPURS IN AN ORCHID

Report

Current Biology

Food Reward Chemistry Explains a Novel Pollinator Shift and Vestigialization of Long Floral Spurs in an Orchid

Graphical Abstract



Highlights

- *Satyrium longicauda* is characterized by two pollination ecotypes
- Pollination by oil-collecting bees is derived from moth pollination
- The pollinator shift process is associated with pre-adaptation and vestigialization
- Minor modifications in floral chemistry can initiate major pollinator shifts

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In Brief

Pollinator shifts were frequent in angiosperms, but how do they happen? Castañeda-Zárate et al. study "evolution-in-action" in an African orchid. They reconstruct a novel shift from moth- to oil-collecting bee pollination. The bee-pollinated form resembles moth-pollinated forms, but produces oil instead of nectar, driving the pollinator shift.

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Report

Food Reward Chemistry Explains a Novel Pollinator Shift and Vestigialization of Long Floral Spurs in an Orchid

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SUMMARY

During the evolutionary history of flowering plants, transitions between pollinator groups (pollinator shifts) have been frequent,[1] and contributed to the spectacular radiation of angiosperms.[2] Although the evolution of floral traits during pollinator shifts has been studied in real time under controlled laboratory conditions,[3] it is challenging to study in nature and therefore poorly understood.[4–7] Using a comparative, multidisciplinary approach, we dissect the evolution of floral traits during a pollinator shift in the long-spurred African orchid *Satyrium longicauda*. Phylogenetic analysis and ecological experiments revealed a shift from moth- to oil-collecting bee pollination. Remarkably, flowers of the bee-pollinated form are similar in morphology, color, and overall volatile chemistry to those of moth-pollinated forms, but differ in having spurs that are mostly devoid of nectar, and have an elevated presence of the oil-derived compound diacetin, which oil-collecting bees use as a cue for oil presence.[8] Experiments demonstrated that long spurs are critical for pollination of a moth-pollinated form, but are not needed for pollination of the bee-pollinated form. We conclude that the pollinator shift in *Satyrium* was mediated by a switch in chemistry of the pollinator reward. The ancestral presence of diacetin might have served as a pre-adaptation for bee pollination, whereas the current mismatch between flower morphology and bees is due to the retention of vestigial floral spurs. These results elucidate the sequence of floral evolution in the early stages of pollinator shifts and help to explain the assembly of suites of co-varying traits through pre-adaptation and vestigialization.[9–12]

RESULTS AND DISCUSSION

Evidence for Pollination Ecotypes in *Satyrium longicauda*

Pollinator shifts occur when populations of the same species adapt to different pollinators.[13] This process results in the formation of pollination ecotypes.[5,14–16] Such ecotypes are characterized by intraspecific variation in floral traits and represent ideal model systems to investigate the evolutionary process underlying pollinator shifts. We discovered the presence of pollination ecotypes in *Satyrium longicauda*, a highly variable southern African grassland orchid that has its center of diversity in a global hotspot of biodiversity.[17] Only two subspecific taxa of *S. longicauda* are currently recognized,[17] but extensive fieldwork revealed the frequent co-occurrence of multiple discrete forms (see below). Studying ecotypes that occur in sympatry offers two great advantages over studying allopatric populations: sympatric co-occurrence (1) minimizes environmental causes of phenotypic differences and reveals their genetic basis[18] and (2) excludes the possibility that observed differences in plant-pollinator interactions reflect geographical turnover in pollinator assemblages[19,20] rather than differences in the function of floral traits. Within a circa 1 km^2 site, we identified six forms of *S. longicauda* that differ in floral traits including spur length and that can be unambiguously diagnosed by the number and position of their leaves and habitat (Figure S1; Table S1). Fruit set was absent when visitors were excluded for all six forms, whereas 70%–100% of cross-pollinated flowers set fruit, indicating pollinator dependence for all forms and confirming previous experiments done on one of the forms.[21]

Previous work has shown that flowers of the two most common forms of *S. longicauda* are pollinated by nocturnal moths.[22] Floral traits of all six forms conform to the syndrome of moth-pollination, including white, sweetly scented flowers with relatively long spurs.[23,24] Our initial hypothesis was therefore that all six forms are pollinated by nocturnal moths, but that variation in spur length could reflect pollination by moths with different tongue lengths.[25] To identify pollinators, we made direct observations and used motion-activated cameras with close-up lenses.[26] Five out of the six forms (*MOTH1–MOTH5*), were visited exclusively or predominantly by nocturnal moths, including hawkmoths (Sphingidae) and settling moths (Noctuidae), which carried pollinaria (Figure 1; Table S2; Video S1). For two of these forms (*MOTH1* and *MOTH3*), occasional visits

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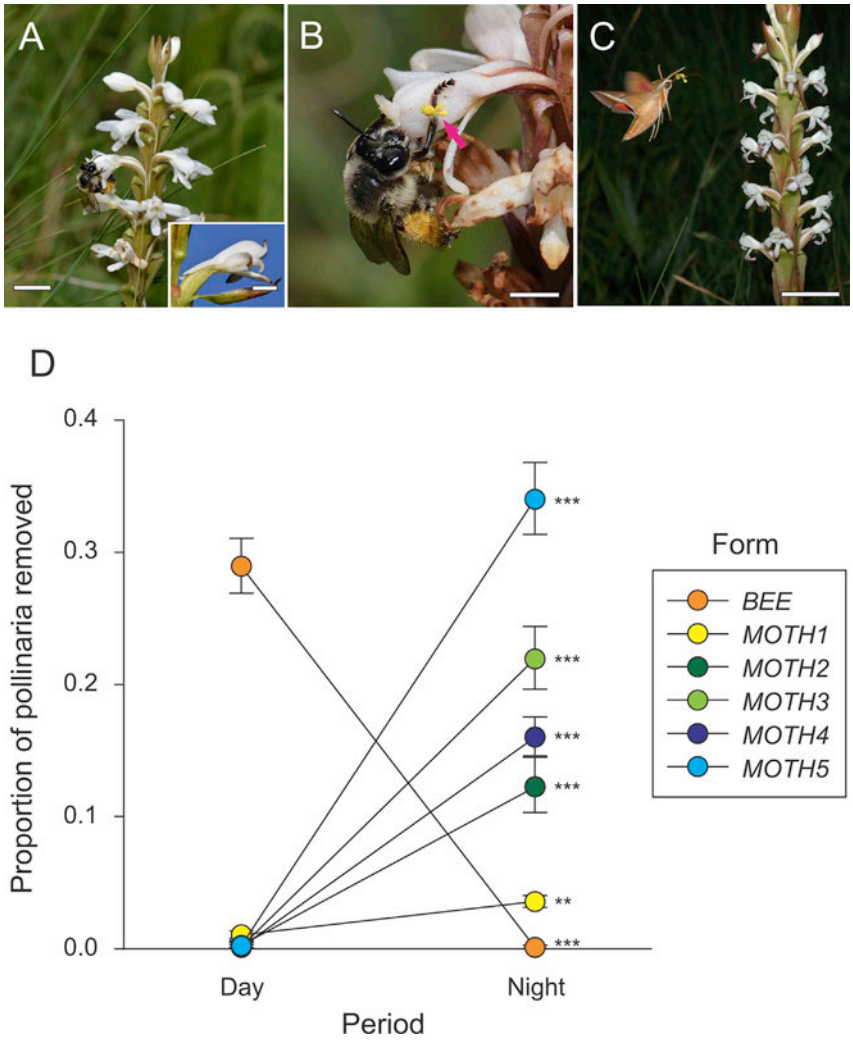

Figure 1. Pollinators of *Satyrium longicauda* and Pollinator Activity Times

(A) An oil collecting *Rediviva neliana* bee visits a flower of the *BEE* form. The inset shows a flower of a rare mutant of the *BEE* form in which floral spurs are almost absent.

(B) *Rediviva neliana* with pollinaria attached to the tibia of its foreleg (pink arrow) and using its other foreleg to collect oil from a flower of the *BEE* form.

(C) The hawkmoth *Basiothia schenki* carries a load of pollinaria on its proboscis as it approaches an inflorescence of the *MOTH4* form.

(D) Comparison of the mean ± SE proportion of flowers with pollinaria removed during day and night periods (form × period $\chi^2_1 = 38.69$, $p < 0.001$)

***$p < 0.001$, **$p < 0.01$. Scale bars, 10 mm (A) and 5 mm (A inset), 5 mm (B), and 50 mm (C). Photo credits: SD. Johnson. See also Table S2 and Video S1.

by diurnal long-tongued nemestrinid flies were also recorded (Table S2). Contrary to our expectations, one form of *S. longicauda* (*BEE*) did not receive any nocturnal visits but was frequently (n = 134) visited by *Rediviva neliana* bees, of which 63.4% carried pollinaria (Figure 1; Table S2; Video S1). All of the 15 *R. neliana* individuals captured while visiting flowers were female, indicating a significant sex bias (exact binomial test: $p < 0.001$). Pollinator behavior and the pollination mechanism differed markedly between *MOTH* and *BEE* forms: moths inserted their proboscides into the spurs of *MOTH* forms while hovering in front of, or settling on, inflorescences, and pollinaria of these forms are attached to the proboscis (Figure 1; Video S1). In contrast, bees did not insert their proboscides into the *BEE* form but rather used their forelegs to probe the galeate labellum. Pollinaria (mean ± SD: 4.0 ± 2.70, range 1–12) were attached to the tibia of bees' forelegs (Figure 1; Video S1) (cf. Pauw[27]). The morphology, site of placement, and angle of orientation of these pollinaria differ from those of all other orchids pollinated by oil-collecting bees that occur in the area.[28]

To confirm the periodicity of pollination (in a manner independent of any observer- or trigger-bias that might influence direct observations and camera trapping, respectively), we also systematically compared pollinarium removal during diurnal and nocturnal intervals.[29] Results showed that pollinarium removal in the five *MOTH* forms is significantly higher at night than during the day, whereas pollinarium removal in the *BEE* form was exclusively diurnal (Figure 1). Natural fruit set among the 6 forms ranged from 79%–90%, indicating that pollinator visits contribute to fecundity.

Contrary to our initial prediction based on floral syndrome traits, these combined results suggest the existence of a bee-pollinated ecotype (*BEE*) that closely resembles the sympatric moth-pollinated (*MOTH*) ecotypes of *S. longicauda*, raising questions about which traits could mediate the partitioning of pollinators among these co-occurring ecotypes.

Functional Traits Associated with Two Pollination Ecotypes

The behavior of moths on flowers was consistent with nectar foraging. The behavior of bees, which involved insertion of their legs rather than their proboscides into flowers, was suggestive of a different foraging mode (Video S1). Female *Rediviva* bees visit flowers not only for nectar, but also to collect oil, which they collect with densely pilose forelegs.[30–32] These floral oils mostly consist of acetylated acylglycerols of fatty acids.[33,34] Observations of foraging behavior (visits exclusively by female *Rediviva* bees, insertion of front legs, extended duration of visits to individual flowers and visits to many flowers per plant) (Video S1), suggest that flowers of the *BEE* form produce floral oil.

To test *S. longicauda* flowers for the presence of oil, we used Sudan IV crystals.[35] As predicted, the inner surface of the labellum and spur entrance of flowers of the *BEE* form stained red, indicating the presence of fatty oil. However, flowers of the five *MOTH* forms stained similarly (Figure S2). To further explore the presence of floral oil-derived compounds, we

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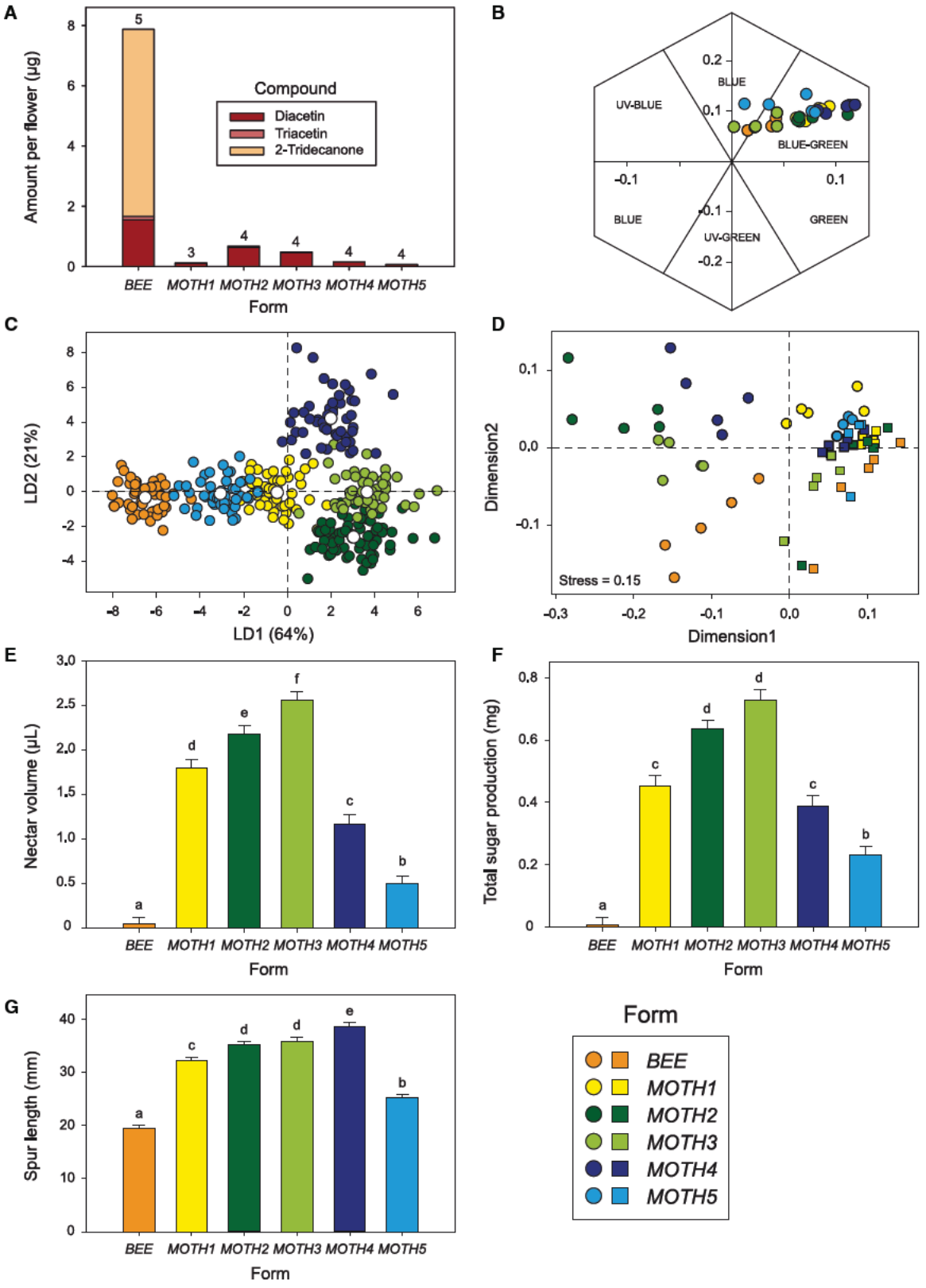

Figure 2. Functional Floral Traits of *Satyrium longicauda*

(A) Production of acetylated glycerols and 2 tridecanone among six forms. Sample sizes are given above each bar.

(B) Flower (labellum) color plotted in the bee vision model, showing that the flower color of the *BEE* form overlaps with that of various *MOTH* forms.

(legend continued on next page)

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analyzed pentane extracts of flowers by using gas chromatography coupled with mass spectrometry (GCMS), to identify the presence of diacetin.[8,36] This volatile acetylated glycerol is thought to be a derivative of the biosynthesis of fatty floral oil, and is considered a universal cue for oil-collecting bees, including *R. neliana*, the pollinator of the *BEE* form.[8] Consistent with the results from staining, GCMS analyses confirmed the presence of diacetin and also triacetin in all six forms (Figure 2). However, quantities were much higher in flowers of the *BEE* form than in the *MOTH* forms (diacetin: $\chi^2 = 113.52$, $p < 0.001$, bee: moth production ratio = 7.83; triacetin: $\chi^2 = 25.731$, $p < 0.001$, ratio = 11.46) (Figure 2). The dependence of *R. neliana* antennal responses on diacetin concentration[8] might explain why bees only visit the *BEE* form of *S. longicauda*. Analyses of floral extracts further revealed the unique presence in the *BEE* form of 2-tridecanone (Figure 2), which is known to trigger antennal responses in oil-collecting bees.[8] Presence of 2-tridecanone is unknown from moth-pollinated *Satyrium* species,[29] but has been reported from the only other—distantly related—*Satyrium* species that is pollinated by oil-collecting bees.[37,38]

To further characterize traits involved in pollinator attraction and morphological fit between flower and pollinator, we quantified color, morphology, and floral headspace volatiles across the six forms. Analysis of spectral reflectance of flowers in a bee vision model showed strong overlap among several *MOTH* forms, but also between the *BEE* form and two *MOTH* forms (Figure 2). A multivariate analysis of 12 quantitative morphometric traits showed that although plants cluster by form (PERMANOVA: $f = 127.36$, $p < 0.001$), there are no clear discontinuities among forms (Figure 2). Furthermore, the mean Euclidean morphological distance between *BEE* and *MOTH* forms was similar to that among *MOTH* forms ($\chi^2 = 0.334$, $p = 0.56$).

Analysis of floral scent headspace sampled during day and night revealed that time of day, form, and the interaction between these factors all differed significantly (2-way PERMANOVA: time of day $f = 45.646$, $p < 0.001$; form $f = 13.556$, $p < 0.001$; time of day × form $f = 6.0347$, $p < 0.001$) (Figure 2). However, similar to the results for morphometric characters, Bray-Curtis similarity of headspace for *BEE* and *MOTH* form comparisons were similar to those among *MOTH* forms, both during day and night (day: $\chi^2 = 0.095$, $p = 0.76$; night: $\chi^2 = 2.64$, $p = 0.10$) (Figure 2; Table S3). A similarity percentage analysis showed that scent across all forms was dominated by aromatic compounds, in particular (E)-cinnamyl alcohol, and that the compounds that dominate the *MOTH* forms also dominate the *BEE* form (Table S3). Several aromatic compounds in the *S. longicauda* headspace are known to elicit antennal responses in the hawkmoth pollinator *B. schenki*.[36]

Given the overall similarity in flower shape, color, and headspace scent, we predicted that the lack of nocturnal moth visitation to the *BEE* form could reflect the lack of a suitable reward. Measurements of nectar volume and sugar concentration confirmed an almost complete absence of nectar in the floral spurs of the *BEE* form, whereas in the *MOTH* forms, nectar volume per flower ranged between 0.5–2.5 μl, resulting in a total sugar availability per flower of 0.2–0.8 mg (Figure 2).

In flowers of the *BEE* form, the near-absence of nectar and the apparent collection of oil by bees from the labellum galea suggest that floral spurs might no longer have a function for pollination. Some oil-producing plants in South Africa produce oil in floral spurs,[39] but the length of their spurs usually closely matches the foreleg length of the pollinating *Rediviva* bees.[40,41] In contrast, the distance from spur tips to the rostellum of the *BEE* form is much longer than the foreleg of local *R. neliana* (mean ± SD functional spur length = 24.00 ± 2.18 mm, n = 50; mean ± SD foreleg length of *R. neliana* females = 10.68 ± 0.58 mm, n = 13), but within the range for moth-pollinated *Satyrium* species (10–24.5 mm), whereas spurs of other bee-pollinated *Satyrium* species are typically shorter (range 5–11.5 mm).[17] Furthermore, the floral spurs of the *BEE* form are much narrower than those of *Diascia* flowers and the only other *Satyrium* species pollinated by oil-collecting bees[38] and thus likely too narrow to accommodate the broad pilose forelegs of *Rediviva* females. In contrast, tongue insertion by moths during nectar foraging indicates a clear function for spurs that is likely to result in strong selection for optimal nectar spur lengths in the *MOTH* forms.[25,42–44] Coefficients of variation (CVAs) for spur length are significantly larger in the *BEE* than in the *MOTH* forms (CVA *BEE* = 11.22; mean ± SD CVA *MOTH* = 7.35 ± 0.73; modified signed-likelihood ratio test (SLRT) statistic = 20.4, $p = 0.0011$), but not for lateral sepal length (CVA *BEE* = 9.25; mean ± SD CVA *MOTH* = 8.56 ± 0.86; SLRT statistic = 4.83, $p = 0.44$), consistent with contrasting signatures of relaxed and strong selection on floral spurs but not other floral traits in *BEE* and *MOTH* forms, respectively (cf. Evans and Bernard[45] and Fenster[46]). The presence, in the population of the *BEE* form, of a mutant that almost completely lacked floral spurs (Figure 1) further suggested relaxed selection on spur length in the *BEE* form. Finally, experimental shortening of floral spurs of the *BEE* and a *MOTH* form confirmed that both pollinarium removal (male fitness component) and massulae deposition on stigmas (female fitness component) were significantly reduced by this manipulation in the *MOTH*, but not the *BEE* form (Figure 3) (cf. Nilsson[42]).

Evolution of Pollination Systems and Floral Traits

Floral traits that do not differ among pollination systems likely did not evolve in association with a pollinator shift. Evolution of such traits might precede a pollinator shift and indicate a pre-adaptation or might represent vestigial structures that have not yet been

(C) Linear discriminant analysis of 12 morphological traits shows that the morphology of the *BEE* form does on average not differ more from *MOTH* forms than *MOTH* forms differ among one another.

(D) Non metric multidimensional scaling analysis of floral scent profiles for all forms suggests that the overall scent profile of the *BEE* form is similar to that of the *MOTH* forms. Circles and squares indicate day and night samples respectively.

(E G) Shown in (E) is mean ± SE nectar volume per flower, in (F) mean ± SE total sugar production per flower, and in (G) mean ± SE spur length (tip to point of fusion with labellum). These three traits all differ significantly among forms (nectar volume: $\chi^2 = 442.09$, $p < 0.001$; sugar content $\chi^2 = 399.51$, $p < 0.001$; spur length: $\chi^2 = 2338.10$, $p < 0.001$).

Letters indicate which forms differ significantly from each other ($p < 0.01$). The legend at the bottom right applies to (B) (G). See also Figure S2 and Table S3.

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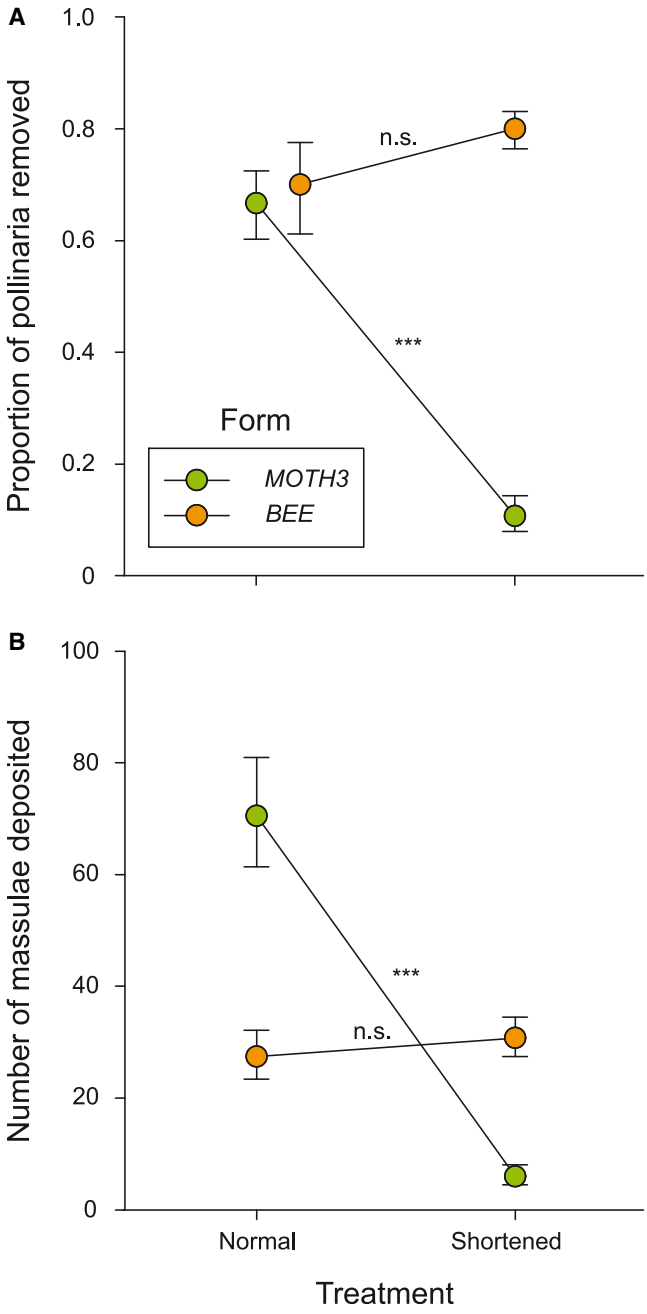

Figure 3. Effect of Spur Shortening on Male and Female Fitness of Two Forms of *Satyrium longicauda* with Contrasting Pollination Systems

Shown in (A) is the mean ± SE proportion of flowers with pollinaria removed (form × treatment: $\chi^2 = 29.83$; $p < 0.001$) and (B) mean ± SE number of massulae deposited on the stigma (form × treatment: $\chi^2 = 50.83$, $p < 0.001$) of the *BEE* and a *MOTH* form. ***$p < 0.001$, n.s., not significant.

lost. To distinguish between these possibilities, we implemented a phylogenetic framework (cf. van der Niet and Johnson[1] and Coddington[47]). A phylogenetic tree based on Bayesian analysis of nuclear DNA sequences of multiple individuals per form supported monophyly of each form, with the exception of *MOTH4*, which was an unresolved polytomy (Figure S3). Together with the morphological clustering of sympatric forms (Figure 2), this suggests that each form represents an independent evolutionary lineage and justifies the application of a phylogenetic approach. The phylogenetic tree based on plastid DNA sequences was less resolved and supported, and partly incongruent with the results from the nuclear data, but also unambiguously supported a sister relationship between the *BEE* and *MOTH1* form (Figure S3).

Ancestral character state reconstruction using parsimony on topologies reconstructed from nuclear and plastid data both showed that pollination by oil-collecting bees in the *BEE* form is derived from moth-pollination in *S. longicauda* (Figure 4). This result was supported by likelihood-based optimization for the nuclear and plastid datasets (Figure S4). Although shifts away from oil production are relatively well-studied,[10,48,49] understanding of the evolutionary origin of pollination by oil-collecting bees was hitherto limited to quantification of the temporal accumulation of lineages and of frequent independent evolution.[49] Previous work on the origin of oil flowers suggested that these might have originated from bee-pollinated ancestors in which pollen and/or nectar was the main reward.[50,51] Some bee-pollinated species jointly produce nectar and oil,[51,52] suggesting a transition through an intermediate stage of double function (cf. Stebbins[13]). Our results thus reveal a novel route for the evolution of pollination by oil-collecting bees.

We propose that the evolution of reward chemistry was key to this unusual transition from moth to bee pollination. The clearly derived nature of bee pollination in this system, combined with the presence of lipids and diacetin in all six forms, suggests that these compounds did not evolve *de novo* in association with the shift to bee pollination. A broader analysis revealed that apart from a record of pollination by oil-collecting *Rediviva* bees in a distantly related *Satyrium* species,[38] no traces of diacetin were found in a sample of *Satyrium* species that includes representatives of all major lineages in the genus.[53] Ancestral character state reconstruction using the topology based on nuclear data suggests that production of detectable amounts of diacetin evolved in the common ancestor of *S. longicauda* (Figure 4). The results based on plastid data suggest the gain of diacetin at the root of the genus with two losses, but this is likely an artifact because of the sparse sampling of species for diacetin in the large clade to which *S. longicauda* belongs[53] (Figure 4). Nevertheless, plastid results and likelihood-based results all imply ancestral presence of diacetin in *S. longicauda* (Figure S4). Diacetin presence in the moth-pollinated forms might have served as a pre-adaptation that triggered occasional visitation by oil-collecting bees (cf. Manning and Goldblatt[51]), followed by additional upregulation and production of 2-tridecanone in response to selection by oil-collecting bees.[8] Presence of diacetin has been reported for another moth-pollinated orchid in a study that also found that moth antennae respond to it.[36] More research is needed to understand the presence of diacetin in species that are not bee pollinated, in order to clarify whether it is a legacy of ancestral pollination by oil-collecting bees or has a functional role in these species.

The shift from moth to bee pollination is associated with a small but significant decrease in spur length (Figure 2). This trend is a departure from the expected unidirectional trajectory of increased spur length evolution[54,55] but could be explained by the vestigialization of floral spurs associated with the shift from

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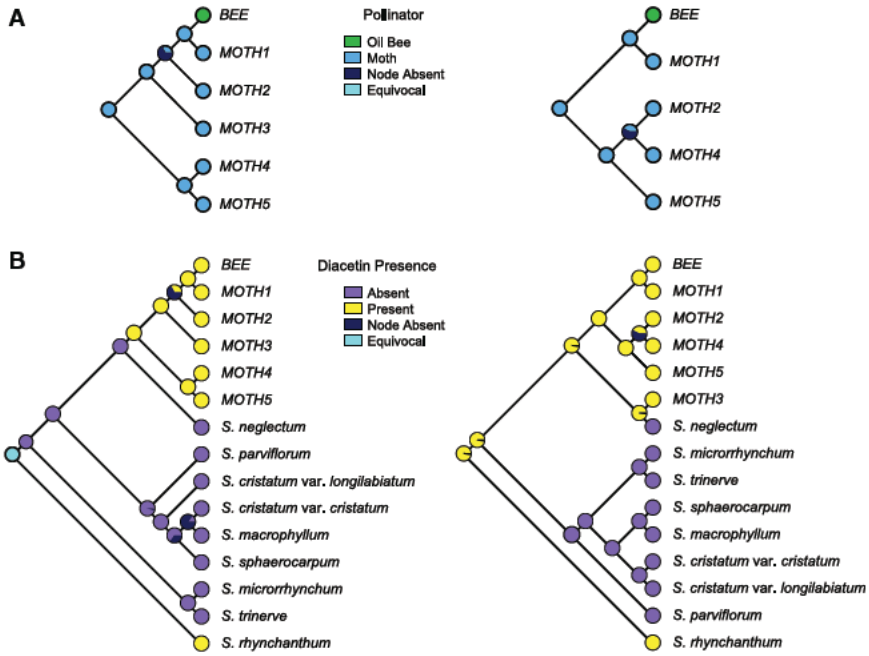

Figure 4. Ancestral Character State Reconstruction

(A) Pollinator (*Satyrium longicauda* complex only).
(B) Diacetin (*Satyrium*).
Results are obtained by using parsimony reconstruction and mapped onto a randomly chosen tree from Bayesian inference of nuclear (left) and plastid (right) DNA sequences. See also Figures S3 and S4.

nectar-foraging moth pollination to oil-collecting bee pollination.[56]

The sympatric ecotypes in *S. longicauda* might represent early stages of speciation, determined by ethological isolation.[57] The existence of multiple moth-pollinated lineages and a single derived *BEE* form would render this a case of progenitor-derivative speciation.[58] Given that the *BEE* form is currently only known from a single site, where it co-occurs with its closest moth-pollinated relatives, the possibility of sympatric speciation cannot be rejected.[59,60] However, in contrast to oceanic islands where historical sympatry is more likely to have been maintained over geological time scales,[60] initial evolution in allopatry, followed by range extension and extinction remains a possibility in this case.[61] Furthermore, the role of additional isolating factors, such as micro-habitat differences, phenological shifts, and post-zygotic isolation need to be investigated to fully understand the strength, nature, and sequence of evolution of reproductive isolation.[62–64]

CONCLUSION

Pollinator shifts have been important for driving extant floral diversity, but the relatively discontinuous nature of suites of floral traits in the currently observable end points of evolution make reconstruction of the underlying evolutionary process challenging. Our study demonstrates the significance of minor modifications in floral chemistry for the initiation of major shifts between insect pollinators belonging to different orders. A similarly pivotal role for minor modifications of floral chemistry has previously been shown in sexually deceptive pollination systems, but these typically involve shifts between closely related pollinators belonging to the same order or even genus.[65–67]

The similarity in floral traits among ecotypes of *S. longicauda* with very different pollination systems, suggests that broadly defined floral syndromes can be inadequate for predicting pollinators, especially if not all traits are functional. Floral spurs in particular are considered a key innovation for angiosperm diversification[68] and their adaptive nature has been emphasized in many studies,[54,55,69] often through covariation between plant and pollinator traits.[70] Our study demonstrates a mismatch between floral spurs and pollinators and indicates that floral spurs can be vestigial. Although vestigialization in plants is well-known from transitions from outcrossing to selfing,[71–73] few studies have combined phylogenetic and experimental approaches to provide evidence for vestigialization during pollinator shifts in entirely pollinator-dependent plants.[10] Our study improves understanding of the sequence of evolution of trait syndromes by providing an example of how divergence in floral rewards could precede that of morphological traits, resulting in a stage of transition characterized by trait vestigialization.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at https://doi.org/10.1016/j.cub.2020.10.024.

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AUTHOR CONTRIBUTIONS

Conceptualization, T.N. and S.D.J; Methodology, T.N., S.D.J., and M.C.Z.; Formal Analysis, M.C.Z, S.D.J., and T.N. jointly analyzed the data; Investigation, M.C.Z.; Resources, S.D.J. and T.N.; Writing, M.C.Z, S.D.J., and T.N jointly wrote the manuscript; Visualization, M.C.Z, T.N., and S.D.J.; Supervision, T.N. and S.D.J.; Project Administration, T.N., S.D.J., and M.C.Z.; Funding Acquisition, S.D.J., M.C.Z., and T.N.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, Peptides, and Recombinant Proteins		
Diacetin (Glycerol diacetate)	Sigma Aldrich	W500615; CAS: 25395 31 7
Tracetin (Glyceryl triacetate)	Sigma Aldrich	90240; CAS: 102 76 1
Pentane	AMD CHROMASOLV®; Riedel de Haën	34894
C7 C40 Saturated Alkanes Standard	Sigma Aldrich, SUPELCO	49452 U
Sudan IV	B. H. D. Laboratory Chemicals Group	NA
Methylene blue	Sigma Aldrich	M9140; CAS: 7220 79 3
Critical Commercial Assays		
DNeasy® Plant Mini Kits	QIAGEN	Cat# 69104
Albumine, Bovine Serum, Fraction V, Fatty Acid Free, Nuclease and Protease Free	Sigma Aldrich, Calbiochem	126609; CAS: 9048 46 8
OneTaq® Quick Load® Master Mix with Standard Buffer	New England Biolabs, Inc.	M0486S
Deposited Data		
Breeding system	This paper	http://doi.org/10.5281/zenodo.4044649
Day/Night pollinaria removals	This paper	http://doi.org/10.5281/zenodo.4044649
Morphometrics	This paper	http://doi.org/10.5281/zenodo.4044649
Natural fruit set	This paper	http://doi.org/10.5281/zenodo.4044649
Nectar volume and concentration	This paper	http://doi.org/10.5281/zenodo.4044649
Scent	This paper	http://doi.org/10.5281/zenodo.4044649
Floral color	This paper	http://doi.org/10.5281/zenodo.4044649
Spur shortening experiment	This paper	http://doi.org/10.5281/zenodo.4044649
ITS alignment	This paper	http://doi.org/10.5281/zenodo.4044649
Plastid alignment	This paper	http://doi.org/10.5281/zenodo.4044649
Software and Algorithms		
Geneious® 10.2.2.	Kearse et al.[74]	https://www.geneious.com/; RRID: SCR 010519
Mesquite 3.61	Maddison and Maddison[75]	http://www.mesquiteproject.org/; RRID: SCR 017994
MrBayes 3.2.7a	Ronquist et al.[76]	https://www.phylo.org/
NIST Spectral Library 2.0 g	Stein et al.[77]	https://www.nist.gov/system/files/documents/srd/NIST1a11Ver2 0Man.pdf
PAST 4.03	Hammer et al.[78]	http://folk.uio.no/ohammer/past/index.html
Primer v6	Clark and Gorley[79]	https://www.primer e.com/
RStudio 3.6.3	Team[80]	https://rstudio.com/; RRID: SCR 000432
R package MASS 7.3 51.5	Venables and Ripley[81]	https://cran.r project.org/web/packages/MASS/index.html
R package cvequality 0.1.3	Krishnamoorthy and Lee[82]	https://github.com/benmarwick/cvequality; RRID: SCR 019124
R package pavo 2.5.0	Maia et al.[83]	https://cran.r project.org/web/packages/pavo/index.html; RRID: SCR 019123
IBM SPSS Statistics 26	IBM	https://www.ibm.com/products/spss statistics?lnk=hpmps bupr&lnk2=learn; RRID: SCR 019096
TNT 1.5	Goloboff and Catalano[84]	http://www.lillo.org.ar/phylogeny/tnt/; RRID: SCR 019122

(*Continued on next page*)

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Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Tracer v1.6	Rambaut et al.[85]	https://bioweb.pasteur.fr/packages/pack@Tracer@v1.6; RRID: SCR 019121
Ocean Optics SpectraSuite SpectroScopy Software	Ocean Optics	https://www.oceaninsight.com/products/software/
Other		
ITS GenBank accessions	This paper	https://www.ncbi.nlm.nih.gov/genbank/ Accession numbers: *BEE* (MT586319 MT586322), *MOTH1* (MT586330 MT586334), *MOTH2* (MT586344 MT586347), *MOTH3* (MT586375 MT586377), *MOTH4* (MT586402 MT586405), *MOTH5* (MT586423 MT586427)
Plastid GenBank accessions	This paper	https://www.ncbi.nlm.nih.gov/genbank/ trn*L* intron: *BEE* (MW053197 MW053200), *MOTH1* (MW053201 MW053205), *MOTH2* (MW053206 MW053209), *MOTH3* (MW053210 MW053212), *MOTH4* (MW053213 MW053216), *MOTH5* (MW053217 MW053221); trn*LF* intergenic spacer: *BEE* (MW053222 MW053225), *MOTH1* (MW053226 MW053230), *MOTH2* (MW053231 MW053234), *MOTH3* (MW053235 MW053237), *MOTH4* (MW053238 MW053241), *MOTH5* (MW053242 MW053246); trn*SG* intergenic spacer: *BEE* (MW053247 MW053250), *MOTH1* (MW053251 MW053255), *MOTH2* (MW053256 MW053259), *MOTH3* (MW053260 MW053262), *MOTH4* (MW053263 MW053266), *MOTH5* (MW053267 MW053271); mat*K*: *BEE* (MW053272 MW053275), *MOTH1* (MW053276 MW053280), *MOTH2* (MW053281 MW053284), *MOTH3* (MW053285 MW053287), *MOTH4* (MW053288 MW053291), *MOTH5* (MW053292 MW053296)

RESOURCE AVAILABILITY

Lead Contact

Further information and requests for resources should be directed to and will be fulfilled by the Lead Contact, Timotheüs van der Niet (vdniet@gmail.com).

Materials Availability

This study did not generate new unique reagents.

Data and Code Availability

Original data have been deposited in Zenodo (http://doi.org/10.5281/zenodo.4044649). DNA sequences can be downloaded from GenBank (https://www.ncbi.nlm.nih.gov/genbank/; ITS accession numbers: *BEE* (MT586319-MT586322), *MOTH1* (MT586330-MT586334), *MOTH2* (MT586344-MT586347), *MOTH3* (MT586375-MT586377), *MOTH4* (MT586402-MT586405), *MOTH5* (MT586423-MT586427). Plastid accession numbers, trn*L* intron: *BEE* (MW053197-MW053200), *MOTH1* (MW053201-MW053205), *MOTH2* (MW053206-MW053209), *MOTH3* (MW053210-MW053212), *MOTH4* (MW053213-MW053216), *MOTH5* (MW053217-MW053221); trn*LF* intergenic spacer: *BEE* (MW053222-MW053225), *MOTH1* (MW053226-MW053230), *MOTH2* (MW053231-MW053234), *MOTH3* (MW053235-MW053237), *MOTH4* (MW053238-MW053241), *MOTH5* (MW053242-MW053246); trn*SG* intergenic spacer: *BEE* (MW053247-MW053250), *MOTH1* (MW053251-MW053255), *MOTH2* (MW053256-MW053259), *MOTH3* (MW053260-MW053262), *MOTH4* (MW053263-MW053266), *MOTH5* (MW053267-MW053271); mat*K*: *BEE*

(MW053272-MW053275), *MOTH1* (MW053276-MW053280), *MOTH2* (MW053281-MW053284), *MOTH3* (MW053285-MW053287), *MOTH4* (MW053288-MW053291), *MOTH5* (MW053292-MW053296).

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Study species and site

Plant species

The experimental subjects were populations of *Satyrium longicauda* Lindl. *Satyrium longicauda* (Orchidaceae) is a terrestrial orchid species, belonging to the subfamily Orchidoideae that is distributed in southern Africa.[17] In South Africa it is a common element of the grassland biome, occurring from near sea level up to 3100 m. Although the current taxonomy recognizes two subspecies,[17] extensive fieldwork has revealed the presence of many distinct forms, which often occur sympatrically. Most of these forms are known from multiple sites and are morphologically and genetically distinct (M.C.Z., unpublished data). Six of these forms co-occur at Mt Gilboa Nature Reserve (South Africa, 29°17'15.32"S, 30°17'34.61"E) (Figure S1). Population sizes of the forms vary from several dozen to hundreds of plants. The first forms start flowering in December, and the last form finishes flowering in April. Several forms co-flower (M.C.Z., unpublished data), but not all forms have overlapping flowering times. In addition to differences in flowering time, there are also differences in micro-habitat among some of the forms, although several forms can be found growing side by side. The main population of the *BEE* form, which is the central focus of this study, does not co-flower with any of the *MOTH* forms that grow in its immediate vicinity, but does overlap in flowering with the *MOTH1* form, which occurs several hundred meters distant (Figure S1).

Fieldwork took place during the flowering seasons of 2017-2019. Voucher specimens of the six forms are lodged in the Bews Herbarium (NU), University of KwaZulu-Natal, Pietermaritzburg campus (Table S1). Ezemvelo KZN Wildlife provided research permits for plant sampling (number OP4624/2018 and OP2302/2019).

Floral visitors

Insects visiting the experimental subjects were caught for identification and presence of pollinaria. Ezemvelo KZN Wildlife allowed insect catching through research permits (number OP2304/2019).

METHOD DETAILS

Pollination ecology

To confirm that all forms of *S. longicauda* need pollinators for fruit set,[21] between 6 (*MOTH3*) and 29 (*MOTH5*) randomly sampled inflorescences of each form were covered with a mesh bag prior to flowering during the flowering seasons of 2017 and 2018 respectively. For each plant, fruit set of a flower that was left unmanipulated was compared to fruit set of a flower that was cross-pollinated.

To characterize the pollination system of each form, we conducted pollinator observations. Observations were carried out while walking through populations of flowering individuals between 08 h00 and 20 h00 during 3-5 warm days and evenings per form. The total number of direct observation h was 84 (*BEE*), 55 (*MOTH1*), 30 (*MOTH2*), 43 (*MOTH3*), 41 (*MOTH4*), and 31 (*MOTH5*) respectively. Visitor behavior was recorded (including an assessment of whether visitors removed pollinaria) and visitors were captured for identification,[86] to measure the length of functional traits (proboscis and foreleg length), and to quantify pollinarium presence. The total number of captured pollinators was 15 (*BEE*), 6 (*MOTH1*), 16 (*MOTH3*), 6 (*MOTH4*), and 23 (*MOTH5*) respectively. No visitors were caught on *MOTH2*. To expand observation h, we also used motion trigger cameras (Bushnell NatureView HD Cam Model #: 119740, USA) to record flower visitors. The reliability of these cameras to record moth foraging activity in *S. longicauda* has recently been demonstrated.[26] This method is not suitable for recording visits by bees as these do not trigger cameras, presumably due to their small size and low temperature contrast between the insect body and relatively high ambient day-time temperatures. The total number of camera h was 360 (*BEE*), 502 (*MOTH1*), 264 (*MOTH2*), 378 (*MOTH3*), 364 (*MOTH4*), and 400 (*MOTH5*) respectively. The number of moth visitors captured on camera ranged from 3 (*BEE*) to 37 (*MOTH4*)

To distinguish whether pollination occurs during the day (consistent with bee pollination) or at night (consistent with moth pollination), we marked the bracts of flowers that had both pollinaria present with a permanent marker on between 10 (*MOTH1*, 2018) and 34 (*BEE*) inflorescences in 2018 (all forms) and 2019 (*MOTH1* and *MOTH2*). For *MOTH1* and *MOTH2* the experiment was run over two years (2018 and 2019), due to low visitation in 2018. Inflorescences were inspected for pollinarium removal at dusk and dawn for three to five consecutive days.[29] In cases where data were available for two years, these were pooled in the analysis (which was supported by a non-significant interaction between the factors 'time of day' and 'year').

Natural fruit set was determined during the flowering seasons of 2018 and 2019, respectively, for each form by counting the total number of fruits out of the total number of flowers produced for 30-50 randomly sampled inflorescences per form that were collected after flowering had finished.

Quantification of floral traits

Floral rewards

The presence of lipids (a constituent of floral oils) was assessed by rubbing small crystals of Sudan IV (B. H. D. Laboratory Chemicals Group, England) along the inner surface of the labellum of at least five flowers of each of the six forms.[35] We further tested for the presence of floral oil indirectly using a comparison of the amount of volatile acetylated glycerols in flowers of the six forms.[8] We immersed whole flowers in 3 mL of Pentane (AMD CHROMASOLV®,[3] 99%, Honeywell Riedel-de HaënTM, Germany) for 1 min to

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obtain floral extracts (cf. Schäffler et al.[8]) from 5 inflorescences of the *BEE* form and 3-4 inflorescences of each of the *MOTH* forms. The same quantity of solvent was placed in empty vials as controls. The description of sample analysis using gas chromatography coupled with mass spectrometry (GCMS) is given below, following the description of the analysis of floral headspace samples.

To quantify differences in rewards among forms, we measured the volume of the nectar standing crop from all six forms of *S. longicauda* by piercing the tip of both spurs (they are too narrow to accommodate a micropipette) and gently drawing their content into a calibrated 5 μL capillary micropipette (ringcaps®, Hirschmann Laborgeräte, Germany). To determine sugar concentration, nectar was transferred to a refractometer designed for small nectar volumes (Eclipse 45–81; Bellingham and Stanley Ltd, Tunbridge Wells, Kent, UK). Sucrose percentage (°Brix) was converted to milligrams of sugar.[87] The number of samples varied among forms: *BEE* = 61, *MOTH1* = 31, *MOTH2* = 42, *MOTH3* = 32, *MOTH4* = 31, and *MOTH5* = 46.

Flower color

To determine whether forms of S. *longicauda* differ in flower color, we measured the spectral reflectance of the outer labellum surface and laterals sepals of five flowers randomly sampled from different individuals for each form. The spectral reflectance from 300 to 700 nm was obtained with an Ocean Optics S2000 spectrophotometer and fiber optic reflection probe (UV/VIS 400 μm). An Ocean Optics Mini DT-2-GS Deuterium–Tungsten–Halogen with a spectral range of 200–2,000 nm was used as a light source. Each measurement was obtained after calibrating the spectra by using a diffuse reflectance standard (Ocean Optics WS 1) followed by spectra capture at 0.38 nm intervals using the Ocean Optics SpectraSuite SpectroScopy Software. For each perianth part the average from two measurements was used for analysis.

Morphology

To determine whether forms differ in morphometric traits, we sampled and measured at least 50 plants of each form (Table S1). A single flower from the middle third of each inflorescence was removed and 12 floral and vegetative traits counted or measured using a pair of digital calipers. The following traits were measured: plant height (from base to tip of inflorescence), inflorescence length, number of flowers, stem base diameter, inflorescence stem diameter at the base, length and width of largest leaf, galea aperture height, galea margin (lip flap) height, lateral sepal length, spur length (distance between the spur tip and the site of fusion with the galea), and functional spur length (spur tip to viscidium).

Floral scent

To quantify and compare scent composition among the six forms of *S. longicauda*, floral headspace samples were collected in the morning (~10 h00) and in the evening (~19 h00), coinciding with the peak of diurnal and nocturnal pollinator activity respectively. Floral headspace samples were collected from five randomly selected flowering individuals of each form in the field or in the laboratory. Sampling was done by placing each inflorescence in a polyacetate bag (Nalophan®, Kalle GmbH, Germany) and pumping the air through an adsorbent trap filled with 1 mg of Tenax® and 1 mg of Carbotrap® at a flow rate of 50 mL min^{-1}. After 30 min of pumping, traps were removed and placed separately inside labeled glass vials and stored at −20°C before analysis. To control for volatiles in the surrounding air, each sampling event also included a sample from an empty bag as described above.

Spur length

We assessed the average spur length among moth- and bee-pollinated *Satyrium* species. Species' pollination systems were based on literature.[22,29,38,88–90] We used the median value of spur length for each species for which pollinator data were available.[17]

To test for a difference in functionality in floral spurs between the *BEE* and a *MOTH* form, we set up an experiment in which we shortened the spurs to half their length. We bagged 20 plants in bud of the *BEE* form in December 2018 and 21 plants of the *MOTH3* form in January 2019 to prevent pollinator access. Once flowers had opened two different treatments were applied. On two flowers spurs were shortened by first gently squeezing them from the tip to move any nectar up, and then folding them at their halfway point. Folded spurs were fixed in that position by using thin (2mm) tape (cf. Ellis and Johnson[56]). A third flower was used as control. Spurs of this flower were not bent, but they were also gently squeezed and fixed with tape to control for manipulation effects. Pollinarium removal and the number of massulae deposited on stigmas were recorded after six days for the *BEE* form, and eight for the *MOTH3* form. Due to some browning of the stigmatic surface a 1% (w/v) solution of methylene blue (Sigma-Aldrich, USA) was used to facilitate counting of massulae.

Phylogenetic inference

DNA sampling and molecular procedure

To reconstruct phylogenetic relationships among the forms of *S. longicauda*, we used an existing framework of species-level phylogenetics that has previously been used in *Satyrium*.[53,91] We used DNA sequences from the nuclear ribosomal internal transcribed spacer (nrITS), which is particularly variable in Orchidaceae and provides resolution at the species level.[92] Given previously detected topological incongruence between datasets from different genomic partitions,[91] we supplemented nuclear data with DNA sequences from the plastid genome, using the *mat*K gene, and the *trn*LF and *trn*SG regions.[93]

We collected foliar tissue from 3-5 individuals of each form and dried this on silica prior to DNA extraction. DNA was isolated using the DNeasy® Plant Mini Kits (QIAGEN, Hilden, Germany). Amplification of the ITS region was carried out using the primers ITS5 and ITS4,[94] whereas the *trn*LF region was amplified using primers trnLF c and trnLF f.[95] The primers trnG and trnS were used to amplify the *trn*SG region,[93] and the plastid gene *mat*K was amplified using the matK −19F and matK R1 primers.[96] The polymerase chain reaction (PCR) for the ITS region was performed using a mix composed of 12.5 μL of OneTaq® Quick-Load® Master Mix with Standard Buffer (New England Biolabs), 1 μL bovine serum albumin (10 ng/μL), 0.5 μL of each primer (100 ng/μl), 10.5 μL distillated H_2O, and 1 μL of extracted DNA (c. 100 ng). Amplification was carried out in a Veriti 96-Well Thermal Cycler following a PCR method that

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included an initial 30 s of denaturation at 94°C, followed by 35 cycles of 30 s denaturation at 94°C, 1 min annealing at 53°C, 1 min extension at 68°C, finished with final extension of 7 min at 68°C. The plastid regions were amplified following protocols used previously in the genus *Satyrium*.[53,91] All PCR reactions were performed in a total volume of 25 μL. The PCR products were purified and sequenced using Sanger sequencing, implementing BigDye chemistry, either at the Central Analytic Facilities (CAF) of Stellenbosch University (South Africa), or at Macrogen Europe (the Netherlands).

QUANTIFICATION AND STATISTICAL ANALYSIS

Timing of pollination

The number of removed pollinaria out of the total number of pollinaria available per flower was compared between day and night periods for each form using generalized estimating equations (GEE) with a binomial distribution and a logit link function in SPSS 26 (IBM Corp.) To control for repeated-measures on the same subject (plant) over time we implemented an autoregressive correlation matrix.[29] We specifically tested whether the timing of pollinarium removal interacted with form (form × time of removal). Estimated marginal means and standard errors were back-transformed from the logit scale, resulting in asymmetrical error bars. Pairwise comparisons were implemented using Sequential Šidák correction.[97]

Floral trait quantification

Rewards

Nectar volume and sugar quantity between forms were both compared using generalized linear models (GLM) with a normal distribution and an identity link function in SPSS 26 (IBM Corp.). The Sequential Šidák correction method was used for multiple pairwise comparisons among forms.[97]

Flower color

For visualization purposes we calculated the color loci of the flower colors in a color hexagon based on honeybee vision.[98] This approach may also provide insight into the question whether the bee species *Rediviva neliana* can discriminate color among forms of *S. longicauda*, as hymenopteran vision systems are relatively conserved.[99] Because no differences in color were observed between the labellum and sepals, we only used the labellum values and plotted these in the bee color hexagon using the R package *pavo* 2.5.0.[83]

Morphology

To visualize differences in morphometric traits between the six forms we implemented a Linear Discriminant Analysis (LDA) on 322 individuals with the *lda* function from the MASS package version 7.3-51.5[81] using RStudio 3.6.3.[80]

To quantify whether morphometric traits vary among forms we performed a one-way permutational multivariate analysis of variance (PERMANOVA) using form as factor. For this analysis we first calculated pairwise Euclidean distances among all individuals of all forms. Because measurements are on a different scale, we standardized the dataset by subtracting the mean and dividing by the standard deviation before calculating Euclidean distance. Distance calculation and PERMANOVA were performed in the software package PAST Version 4.03.[78]

To determine whether the *BEE* form differs more from *MOTH* forms than *MOTH* forms do from each other, we contrasted mean pairwise comparisons of morphological distance. Distances were averaged for comparisons between individuals of each possible pairwise comparison between forms. This resulted in five comparisons between the *BEE* and five *MOTH* forms, and ten comparisons among the five moth forms. We then used GLM with a Gaussian distribution and an identity link function in SPSS 26 (IBM Corp.) to compare whether pairwise differences vary for 'between pollination system' versus 'within pollination system' comparisons.

Chemical analysis

Volatiles were characterized and quantified using GCMS. Traps were analyzed using a Varian CP-3800 gas chromatograph (Varian, Palo Alto, California, USA) with an Alltech EC-WAX column (30 m × 0. 25 mm internal diameter × 0.25 μm) coupled to a Bruker 300-MS quadrupole mass spectrometer in electron-impact ionization mode. Traps were placed in a Varian 1079 injector equipped with a Chromatoprobe thermal desorption device.[100] The flow of helium carrier gas was 1 mL min^{-1}. For thermodesorption, the injector was held at 40°C for 2 min with a 20:1 split and then increased to 200°C at 200°C min^{-1} in splitless mode. Meanwhile, the gas chromatograph oven was held at 40°C for 3 min and then ramped up to 240°C at 10°C min^{-1} and held there for 12 min.[29] Quantification of emission rates was done by injecting a known amount of methyl benzoate and run it using the same temperature program. The resulting peak area in the chromatogram was calibrated and compared to the total peak area of samples.[29]

Compound identification was done by comparing mass spectra and the Kovats Retention Index (based on comparison of compound retention times to those of a set of alkanes) to published values using the Bruker Workstation software Version 7.0 in combination with the NIST Mass Spectral Program for the NIST Spectral Library Version 2.0 g.[77] This approach resulted in identification of most peaks. Peaks that could not be identified were scored as unknown, with the six most dominant mass fragments indicated. Quantification of compounds was based on integrating the area under peaks in chromatograms.

Comparison of scent samples taken among forms during both day and night was done using 2-way PERMANOVA in the software PAST 4.03,[78] testing for an effect of time of sampling (day/night), form, and the interaction between these two factors. The comparison of differences among and within pollinator group comparisons followed the same procedure as was done for the morphometrics dataset, although this test was done separately for the set of samples taken during day and evening respectively. To compare scent profiles of samples we first applied square-root transformation to the proportion of identified compounds, to downweight the

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influence of dominant compounds, and then calculated Bray-Curtis similarity. For the comparison of average Bray-Curtis similarity between *BEE* and *MOTH* forms versus among *MOTH* forms, we logit-transformed the average Bray-Curtis similarity as these values are bounded between 0 and 1. For visualization of variation among samples we plotted these using non-metric multidimensional Scaling (NMDS) in PAST 4.03.[78]

To quantify which compounds are most characteristic for each form, we used a similarity percentage (SIMPER) analysis, focusing on compounds that had a cumulative contribution to 50% similarity. This analysis was done for day and evening samples separately, based on Bray-Curtis similarity of square-root transformed proportions of compounds in the software Primer v6.[79]

The presence of volatile acetylated glycerols was quantified by placing 5 μL of floral pentane extract in a quartz vial and inject this into a SCION 436-GC with a SGE SolGel Wax polar column (30 m × 0.25 mm internal diameter × 0.25 μm film thickness) coupled to a SCION SQ (Livingston, UK) using the same temperature method as was used for the headspace samples, but with an additional back-up step holding the temperature at 260°C during 25 min at 20°C min^{-1} to bake out the column to remove possible residual compounds that are part of the direct flower extract. Acetylated glycerol compounds were identified and quantified based on comparison with synthetic standards. For both diacetin and triacetin, 2 μL was placed in a quartz vial and injected under identical conditions as described above. The chromatograms were used to calculate peak area per mass. This information was used to calculate how much of each of these two compounds was present per flower. Other compounds associated with oil-producing flowers were identified based on their highly characteristic mass spectrum and Kovats Retention Indices that were calculated against a set of alkanes run under the same temperature conditions using the MS Workstation software Version 8.0.1 with the NIST Mass Spectral Program for the NIST Spectral Library Version 2.0 g.[77] The quantity of other compounds was calculated by averaging the peak area per mass for diacetin and triacetin as a reference. None of the control samples that were obtained without flowers showed any signs of acetylated glycerols or other compounds related to oil production.

To compare amounts of volatiles associated with oil production, we integrated the area under each peak and compared the quantity between the *BEE* and *MOTH* forms. This comparison was done using GLM in SPSS 26 (IBM Corp.) implementing a Gamma distribution with a log link function, with pollinator as predictor and form nested inside pollinator. For two samples of the moth forms, no triacetin was detected. To allow the model to run we replaced a 0 with a value of 1.0^{-10}, which makes the test slightly more conservative.

Spur length variation and functionality

To test whether spur length (measured from the tip to where they merge with the labellum) varies among forms, we used GLM implementing a Gaussian distribution with an identity link function. Pairwise comparisons were done by implementing the Sequential Šidák procedure in SPSS 26 (IBM Corp.).[97]

To test whether the coefficient of variation in spur length is greater in the *BEE* form than the *MOTH* forms (as a signature of relaxed selection) we calculated a coefficient of variation of spur length based on the measurements described above and compared these using the modified signed-likelihood ratio test[82] in the R package *cvequality* version 0.1.3.[101] To assess whether the observed pattern is unique for spur length (specifically indicating relaxed selection in the *BEE* form for this particular character), we repeated this analysis for the character lateral sepal length.

To test for an effect of spur bending on male fitness (pollinarium removal) and female fitness (number of massulae deposited on the stigmas), we analyzed the data using GEE with plant as a subject. For pollinarium removal (number of pollinaria removed out of number present) we implemented a binomial distribution with logit link function and for number of massulae deposited we implemented a negative binomial distribution with a log link function in SPSS 26 (IBM Corp.). We tested whether there was an interaction between treatment and form, and compared marginal means within forms using the Sequential Šidák procedure.[97] Estimated marginal means and standard errors were back-transformed to the original scale and plotted as asymmetrical error bars.

Phylogenetic analyses and ancestral character state reconstruction

Sequence alignment and phylogenetic analyses

DNA sequence electropherograms were edited and assembled with Geneious® 10.2.2.[74] Sequences were then aligned by eye with the matrix previously used for a species-level phylogenetic analysis of *Satyrium*.[91] Phylogenetic relationships were inferred from nuclear and plastid sequences separately through maximum parsimony and Bayesian Inference. The parsimony search started with 10,000 random-addition sequence replicates of Wagner trees, retaining 100 trees per replication, followed by tree bisection-reconnection (TBR) branch swapping, performed in TNT 1.5.[84] Bootstrap resampling was used to evaluate support of the nodes of the most parsimonious tree.[102] Results of 10,000 replicates were summarized using absolute frequencies for each group. Bayesian analyses were conducted in the program MrBayes version 3.2.7[76] on the CIPRES Science Gateway using default settings. The evolutionary models were selected according to a previous analysis.[91] Searches consisted of five million generations with chain sampling every 1,000 generations. The first 20% of generations was discarded as burn-in. Convergence was confirmed by evaluating whether the Effective Sample Size of all estimated parameters was above 200 in Tracer v1.6.[85] After removing the burn-in generations, each 8^{th} tree was selected from each of the two treefiles and combined for a dataset of 1,000 trees that was used for ancestral character state reconstruction.

Ancestral character state reconstruction

To polarize the direction of the pollinator shift and reconstruct the evolution of floral oil we implemented ancestral character state reconstruction. Prior to this we omitted taxa not relevant for this analysis. The final matrix included all *S. longicauda* accessions from Mt Gilboa, as well as a further seven species of *Satyrium*, sampled for the presence of acytelated glycerols (see below), which

were chosen as representatives of the main clades of *Satyrium* (see Figure S3 and[91] for information on GenBank accession numbers). These species include the two varieties of *S. cristatum*, *S. macrophyllum*, *S. microrrhynchum*, *S. neglectum* ssp. *neglectum*, *S. parviflorum*, *S. rhynchanthum*, *S. sphaerocarpum*, and *S. trinerve*. In case multiple accessions were available for a species or form, we randomly selected a single individual per form or species per generation from the set of 1,000 trees for inclusion in the trees used for ancestral character state reconstruction. We performed both parsimony and likelihood-based analyses of ancestral character state reconstruction, but focus mainly on the parsimony results using Fitch parsimony.[103] The rationale is that under certain conditions, especially if evolutionary change is infrequent, model-based approaches can return counter-intuitive results (see Figure 3 in Pagel[104]). A preliminary analysis implementing Bayesian ancestral character state reconstruction confirmed that our dataset is similar to the case discussed in Pagel.[104] In such cases, parsimony may outperform model-based approaches.[105] Furthermore, model-based approaches are particularly useful in datasets with long branches (where multiple changes per branch, which cannot be modeled with parsimony, are expected). However, our focus is on reconstructing character evolution within a recently evolved species complex, where branches are relatively short. We first reconstructed the ancestral pollinator in the *S. longicauda* species complex. We coded each form by its primary pollinator (moth versus oil-collecting bee). We then performed ancestral character state reconstruction on the set of trees retrieved from the Bayesian phylogenetic analysis using the 'Trace Character over Trees' command in Mesquite 3.61.[75] These analyses were done separately for the trees resulting from the phylogenetic analysis of plastid and nuclear DNA sequences respectively. For the plastid analysis, one of the *MOTH* forms was excluded for this analysis, as it was not part of the clade that comprises the *BEE* form and most other *MOTH* forms. We performed likelihood-based analyses in Mesquite, using the MK1 model.[104]

We also traced the evolution of floral oil in *Satyrium*. For this analysis we first took floral extracts of six of the seven species mentioned above (apart from *S. rhynchanthum*) according to the methods described above for *S. longicauda*. We then coded each species for oil presence or absence. For *S. rhynchanthum*, which is pollinated by *Rediviva gigas*,[38] we confirmed the presence of diacetin in a floral headspace sample (T.N., unpublished results). The evolution of floral oil was analyzed using the same method as described for pollinator.

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Supplemental Information

Food Reward Chemistry Explains a Novel Pollinator Shift and Vestigialization of Long Floral Spurs in an Orchid

Miguel Castañeda-Zárate, Steven D. Johnson, and Timotheüs van der Niet

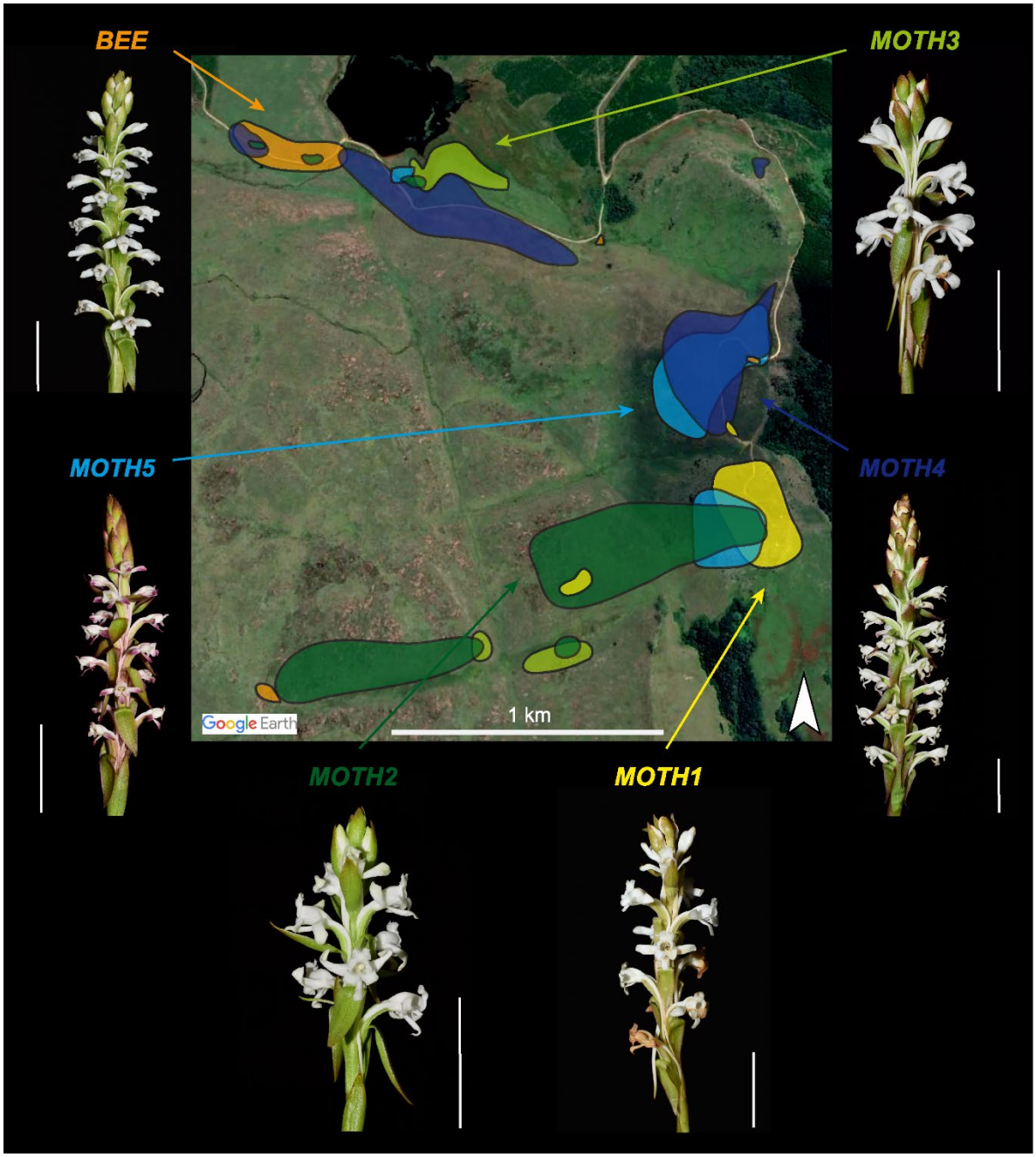


Figure S1. Map with approximate distribution of the six forms used in this study.

Related to STAR methods. Scale bar = 50 mm.

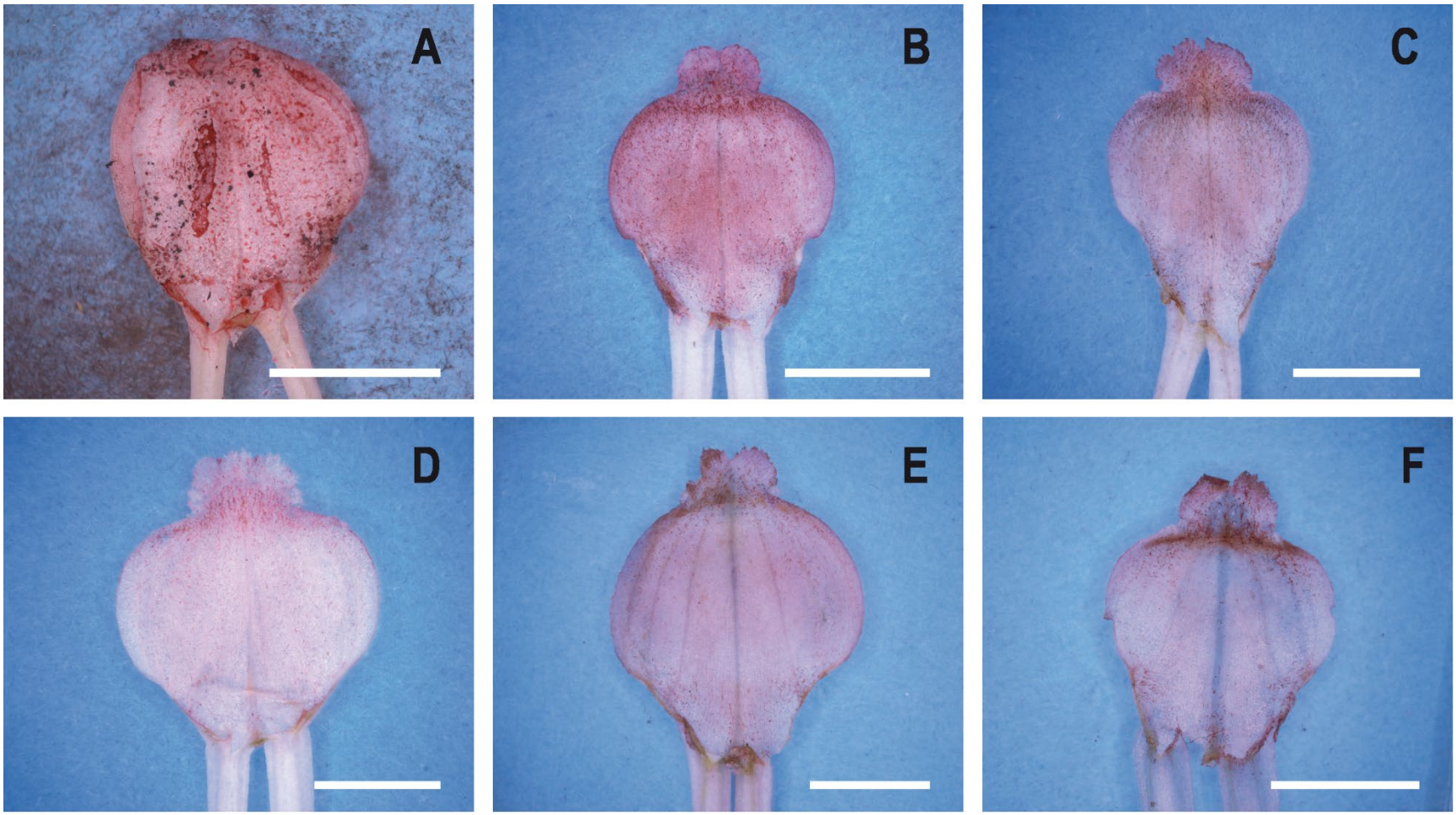


Figure S2. Inside labellum surface stained with Sudan IV crystals. Related to Figure 2. (A) *BEE*, (B) *MOTH1*, (C) *MOTH2*, (D) *MOTH3*, (E) *MOTH4*, (F) *MOTH5*. Scale bar = 5 mm.

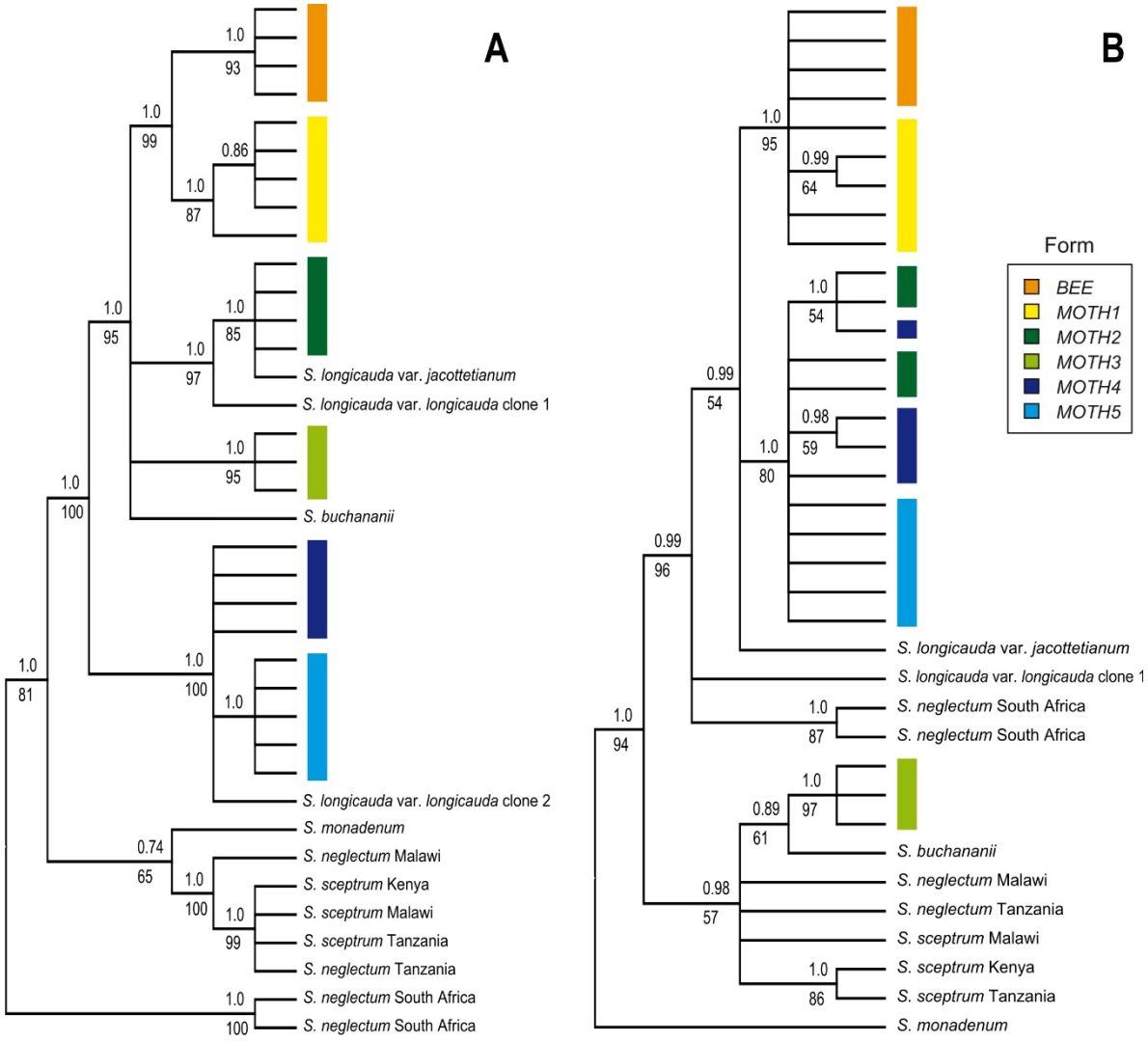


Figure S3. Majority rule consensus trees of Bayesian inference of *Satyrium longicauda* and closely related taxa based on (A) nuclear and (B) chloroplast DNA sequences.

Related to Figure 4. Parsimony bootstrap values are given above branches, Bayesian posterior probability values are given below the branches. Taxon names refer to [79]. The *BEE* form is coded in orange and is well-supported to be sister to *MOTH1* in both topologies.

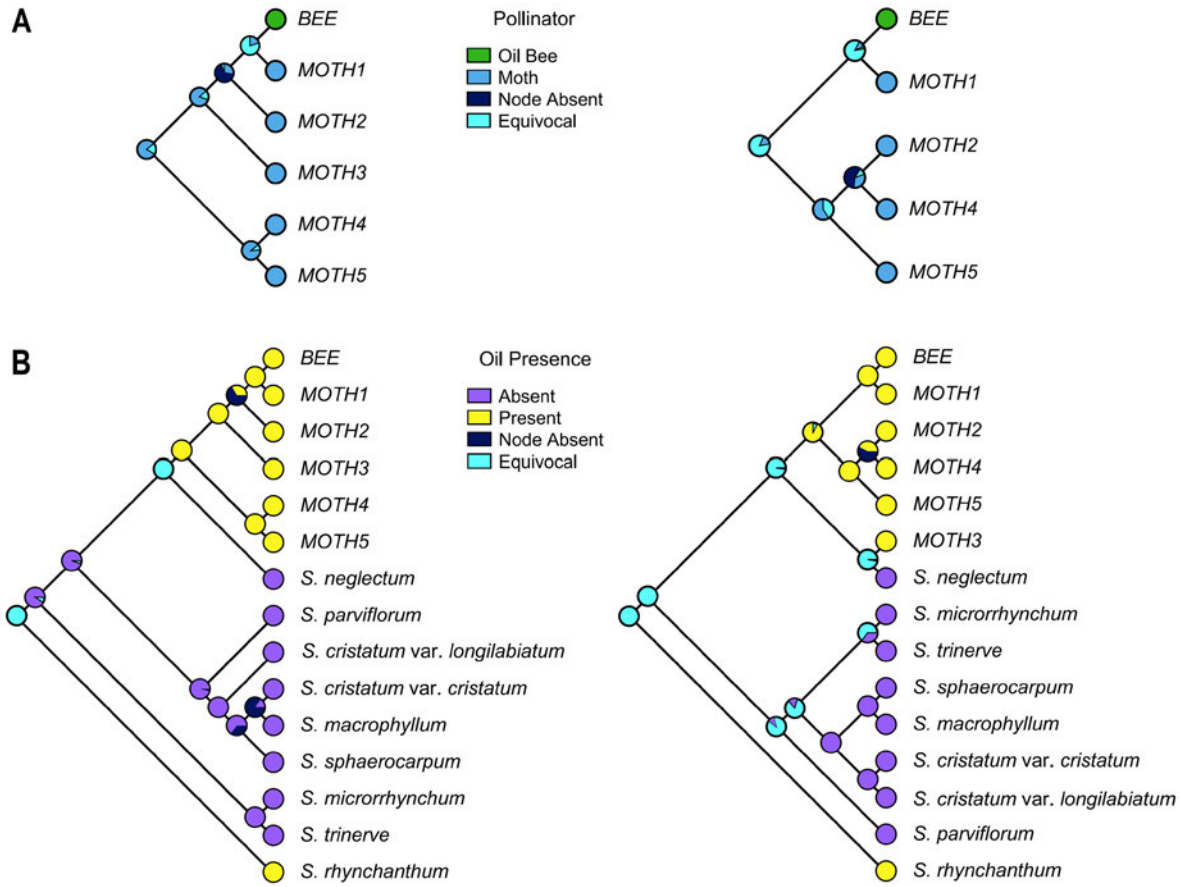


Figure S4. Ancestral character state reconstruction of (A) pollinator (*Satyrium longicauda* complex only) and (B) diacetin (*Satyrium*). Related to Figure 4. Results are obtained using maximum likelihood reconstruction and mapped onto a randomly chosen tree from Bayesian inference of nuclear (left) and plastid (right) DNA sequences.

Species	Form	N (n)	Collection number
Satyrium longicauda Lindl.	*BEE*	50 (5)	1412, 1414, 1488, **1494**, 1606, 1614
	MOTH1	50 (3)	1490, **1495**, 1612, 1622
	MOTH2	53 (4)	1413, 1508, **1613**
	MOTH3	69 (4)	1415, 1426, **1523**, 1620, 1635
	MOTH4	50 (4)	1425, 1444, **1537**, 1547, 1634
	MOTH5	50 (4)	1443, 1452, 1453, **1641**, 1657
Satyrium cristatum Sond. var. *cristatum*		1	**1557**
Satyrium cristatum var. *longilabiatum* A.V. Hall		1	**1528**
Satyrium macrophyllum Lindl.		3	**1556**, **1567**, **1568**
Satyrium microrrhynchum Schltr.		2	**1549**
Satyrium neglectum Schltr.		2	**1530**, **1553**
Satyrium parviflorum Sw.		2	**1524**, **1531**
Satyrium sphaerocarpum Lindl.		1	**1534**
Satyrium trinerve Lindl.		1	**1554**

*Samples were collected by the first author under the acronym of MCZ. Voucher specimens are housed in the UKZN's Bews Herbarium (NU), Pietermaritzburg, South Africa.

Table S1. Voucher table of *Satyrium longicauda* forms and other *Satyrium* species measured for acytilated glycerols. Related to STAR methods. Sample sizes of individuals measured for morphometrics and oil presence (between brackets) are provided. Collection numbers in bold refer to the collection used to obtain floral solvent extracts.

Form	Species	Individuals observed directly (number with pollinaria)	Individuals recorded on camera (number with pollinaria)	Mean number of pollinaria (range)	Proboscis length (mean±SD)
BEE	**HYMENOPTERA**				
	Melittidae				
	Rediviva neliana Cockerell	134 (84)	2 (1)	4 (1-12)	—
	Apidae				
	Xylocopa sp.	1 (0)	—	—	—
	LEPIDOPTERA				
	Pieridae				
	Colotis eris Klug	1 (0)	1 (0)	—	—
	Nymphalidae				
	Unidentified	1 (0)	—	—	—
	Noctuidae				
	Unidentified	1 (0)	—	—	—
MOTH1	**LEPIDOPTERA**				
	Sphingidae				
	Basiothia schenki (Möschler)	—	3 (1)	—	—
	Hippotion celerio (Linnaeus)	1 (1)	—	—	—
	Noctuidae				
	Cucullia hutchinsoni Hampson	3 (1)	—	5	32.79
	Cucullia terensis Felder & Rogenhofer	1 (0)	—	—	28.4
	DIPTERA				
	Nemestrinidae				
	Prosoeca sp.	6 (4)	—	1.17 (1-3)	25.1
	Tabanidae				
	Philoliche aethiopica Thunberg	1 (1)	—	1	15.8
MOTH2	**LEPIDOPTERA**				
	Sphingidae				
	Basiothia schenki (Möschler)	2 (1)	3 (2)	—	—
	Noctuidae				
	Cucullia hutchinsoni Hampson	1 (1)	1 (0)	1	—
MOTH3	**LEPIDOPTERA**				
	Sphingidae				
	Basiothia schenki (Möschler)	87 (27)	7 (5)	3.17 (1-7)	40.68±1.24
	Hippotion celerio (Linnaeus)	1 (1)	1 (1)	—	—
	Hippotion osiris (Dalman)	—	1 (1)	5	—
	Noctuidae				
	Cucullia hutchinsoni Hampson	20 (5)	8 (2)	2.70 (1-6)	32.4±1.27
	Cucullia terensis Felder & Rogenhofer	1 (0)	—	—	26.9
	Pieridae				
	Colotis eris Klug	1 (0)	—	—	—
	DIPTERA				
	Nemestrinidae				
	Prosoeca sp.	5 (0)	—	—	26.45±3.00
MOTH4	**LEPIDOPTERA**				
	Sphingidae				
	Agrius convolvuli (Linnaeus)	2 (1)	—	1	91.22
	Basiothia schenki (Möschler)	59 (27)	26 (18)	2.40 (1-7)	41.08±2.63
	Noctuidae				
	Cucullia hutchinsoni Hampson	20 (13)	11 (3)	—	—
	Unidentified	1 (0)	—	—	—

	LEPIDOPTERA				
	Sphingidae				
	Basiothia schenki (Möschler)	1 (0)	—	—	—
	Hippotion eson (Cramer)	1 (0)	—	—	45.08
	Hyles livornica (Esper)	1 (1)	—	1	28.81
MOTH5	Noctuidae				
	Cucullia chrysota Hampson	2 (1)	—	4	26.78±5.96
	Cucullia hutchinsoni Hampson	70 (41)	14 (9)	2.57 (1-4)	31.88±1.70
	Cucullia terensis Felder & Rogenhofer	2 (1)	—	4	28.01±0.71
	Thysanoplusia orichalcea (Fabricius)	2 (1)	—	1	16.16±1.87
	Unidentified	1 (0)	—	—	17.41
	Unidentified	1 (0)	—	—	7.21

Table S2. Insects observed visiting *Satyrium longicauda.* Related to Figure 1. Visitors are grouped by family within their respective order. Proboscis length is given in millimetres.

CHAPTER 3

CONGRUENT MORPHOLOGICAL AND GENETIC DATA REVEAL OVERLOOKED SYMPATRIC MORPHOTYPES IN THE *Satyrium longicauda* COMPLEX (ORCHIDACEAE)

Abstract

Delimitation of species boundaries when there is a high degree of morphological variation or a wide geographical distribution represents a taxonomical challenge, especially if taxonomy is primarily based on herbarium collections. The orchid *Satyrium longicauda* combines these features and currently two varieties, var. *longicauda* and var. *jacottetianum,* are accepted, based mainly on differences in the length of the lateral sepal and nectar spur. However, there is extensive morphological variation within South African populations and evidence for several pollination ecotypes, indicating that this taxon represents an actively diverging species complex. Here, we evaluate intraspecific morphological variation through multivariate statistics and analyse genetic diversity using sequences from the internal transcriber spacer (ITS) for individuals sampled from 36 sites, including 14 sites where divergent morphotypes occur sympatrically. Morphometric analyses confirmed the presence of eight discrete morphotypes based on unique combinations of vegetative and floral characters. Up to six morphologically and genetically distinct morphotypes can co-exist within sites of just a few hectares. Congruence between morphological and genetic distances was confirmed by a significant correlation. Phylogenetic analyses indicated that neither of the two currently recognised varieties, nor *S. longicauda* as a species are monophyletic, but provided evidence for the monophyly of some of the morphotypes. Further geographical sampling is required to inform taxonomic decisions but our results confirm that taxonomy mainly based on herbarium collections can grossly underestimate actual diversity of disparate lineages. These findings have implications for efforts to estimate species diversity in groups that are in the process of diversifying, and have consequences for conservation practice.

Key words: internal transcribed spacer (ITS), multivariate statistics, species complex, sympatry, taxonomy

Introduction

Species represent the central units of study in biology, and taxonomy is recognised as the starting point for other disciplines in which species are the object of study (De Moraes 1987). Three different, but related activities in taxonomy are to delimit, describe, and identify species (Cook *et al.* 2010). Decisions about species delimitation are ultimately implicitly or explicitly guided by author bias, methodology, and the species concept used (Sites and Marshall 2004; de Queiroz 2007; Devey *et al.* 2008; Lumbsch and Leavitt 2011; Shapiro *et al.* 2016; Spooner 2016). Taxonomists often use a species concept that defines species based on discrete morphological variation (Balakrishnan 2005; Ezard *et al.* 2010; Koffi *et al.* 2010). Specifically, the Typological (or Essentialist, Morphological, or Phenetic) Species Concept considers organisms as part of the same group based on the degree of morphological similarity. However, this species concept has been criticised as it does not take the evolutionary process underlying the formation of species (speciation) into consideration (Mayr 1992, 2000; Rapini 2004; Harper *et al.* 2009; Koffi *et al.* 2010). The most influential species concept among biologists who work in other fields, including evolutionary biology, is the Biological Species Concept (BSC; Velasco 2008; Hausdorf 2011), which defines species as groups of actually or potentially interbreeding populations, which are reproductively isolated from other such groups (Mayr 1942). It thereby views reproductive isolation as the key criterion for defining species boundaries. However, the BSC or any other species concept that considers species to be independent lineages still requires that species be identifiable and also usually accepts that distinct morphological features are emergent properties of species, and as such it is possible to make dual use of both morphological and evolutionary species concepts. Taxonomic stability is reached if different operational species concepts converge on a similar number and delimitation of taxa.

The central criterion of the BSC for delimiting species is whether they are reproductively isolated (Wu 2001; Sobel *et al.* 2010; Shaw and Mullen 2011). This may be difficult to establish among allopatric populations, but sympatric populations provide a direct test of the BSC: if assortative mating occurs, populations may be considered different species (Devey *et al.* 2008; Xu *et al.* 2011; Ikabanga *et al.* 2017). Although it may be challenging to quantify mating patterns, assortative mating is expected to result in discrete morphological and genetic variation among sympatric populations as opposed to admixing that occurs in hybrid zones. In cases where species maintain their integrity in sympatry, the presence of morphological variation and reproductive isolation are thus linked (Wojcieszek and Simmons 2013; Queiroz *et al.* 2015), resulting in congruence between species delimitation according to the Biological and Typological Species Concept (Waters *et al.* 2001; Koffi *et*

al. 2010). However, sympatric variants may also represent variation within an interbreeding population, in which case discrete morphological units are not necessarily genetically distinct (Jersáková *et al.* 2010; Breitkopf *et al.* 2013). A key issue is therefore to analyse morphological and genetic patterns of within-site variation.

Classical taxonomic investigations are typically based on the evaluation of herbarium collections (Henderson 2005, 2006). Since collectors usually collect one or few individuals per site, identification of variation that may exist at a local scale remains unrecognised and thereby precludes proper identification of sympatric variants and statistical analysis of morphological discontinuities within populations. In addition, specimens often lack information for some key traits, or characters may be hidden or altered due to the pressing of specimens (Henderson 2006; Koffi *et al.* 2010; Tomaszewski and Górzkowska 2016; Botes *et al.* 2020). These factors together may result in an underestimation of the true levels of biodiversity. The study of morphological variation based on detailed sampling of fresh material at the population level of sympatric morphotypes may reveal characters and patterns not observed on dried plant herbarium specimens (Ikabanga *et al.* 2017; Prata *et al.* 2018; Lissambou *et al.* 2019). Using multivariate analyses of morphological data facilitates the identification and selection of diagnostic morphological characters (Henderson 2006; Marhold 2011). The further inclusion of molecular techniques can be used to assess whether morphological variants are genetically distinct, thereby contributing to quantification of reproductive isolation (Østbye *et al.* 2005; Lowry *et al.* 2008; Xu and Schlüter 2015; Prata *et al.* 2018). Genetic data can provide additional insight into the evolutionary history and phylogenetic relationships (Cook *et al.* 2010; van Velzen *et al.* 2012; Larranaga *et al.* 2019). Phylogenetic information can also provide insight into species monophyly if sampling is sufficient. Testing monophyly of species can reveal insight into the speciation process. For instance, genetic data have been used to identify patterns of progenitor-derivative speciation (Schlüter *et al.* 2011), in which rare neo-endemics budded off from a widespread paraphyletic species (Anacker and Strauss 2014).

Satyrium longicauda Lindl. is a member of the largely African orchid genus *Satyrium*, species of which are characterised by having non-resupinate flowers with a hooded labellum from which two spurs project downward (Hall 1982; Kurzweil and Linder 1999, 2001). The species is a common element of Southern African grasslands and is distributed from Malawi in the north to South Africa in the south. In his revision of the Southern African species of *Satyrium*, Hall (1982) identified *S. longicauda* as extensively variable. Based on a detailed analysis of measurements of various floral and vegetative characters taken predominantly from herbarium specimens and, to a lesser degree,

fresh material, he found evidence for the existence of three taxa based on overall size variation, which could be diagnosed by differences in nectar spur and lateral sepal length (Hall 1982). Although he also suggested that sympatric variants he had observed, which differed in certain traits such as leaf number and position (that he considered likely the result of single gene polymorphisms), could be recognised at the forma level, he followed a more conservative approach leading to the recognition of two varieties within *S. longicauda*: var. *longicauda* Lindl. and *var. jacottetianum* (Kraenzl.) A.V. Hall. The latter was based on a description of *Satyrium jacottetianum* by Kraenzlin (1915). Hall (1982) did not recognise *Satyrium buchananii*, described by Schlechter (1897), as a third variety of *S. longicauda* because of a shortage of available material and because it fell outside the geographical distribution considered for his revision.

Hall's (1982) taxonomy has not been formally evaluated to date. However, several studies have shed some light on the taxonomy of *S. longicauda*. In a recent study focusing on the evolution of pollination systems, Castañeda-Zárate *et al.* (2021) studied six sympatric morphotypes of *S. longicauda* and showed that these were morphologically and genetically distinct (Chapter 2). However, this study only included representatives from a single site and is therefore not representative for variation across the species range. Species-level phylogenetic analyses clarified the relationships within *Satyrium* (van der Niet *et al.* 2005, 2011) and showed that *S. buchananii* is nested inside three Operational Taxonomic Units (OTUs) of *S. longicauda* based on nuclear ITS sequences (van der Niet and Linder 2008; van der Niet *et al.* 2011). Although this inference was based on a limited number of samples, the results suggest that *S. longicauda sensu stricto* is not monophyletic (van der Niet 2017). Another taxon for which its affinities to *S. longicauda* are unclear is *S. rhodanthum* Schltr. The highly divergent traits that characterise this taxon reflect its unusual bird pollination system (van der Niet *et al.* 2015) and it was previously considered a putative hybrid between *S. longicauda* and *S. neglectum* ssp. *woodii* (Hall 1982). However, *S. rhodanthum* has been treated as a species in more recent taxonomic treatments (Linder and Kurzweil 1999; Johnson and Bytebier 2015).

In the course of recent extensive fieldwork in South Africa, which is the centre of diversity for *S. longicauda* (Hall 1982), we detected the presence of what appeared to be multiple distinct morphotypes which often grow together and bloom at the same time, but that differ in floral morphology and the arrangement of the leaves (Castañeda-Zárate *et al.* 2021; Chapter 2). The diversity of morphological units appeared to be even more pronounced than those noted in Hall's earlier observations (Hall 1982). Given the broad geographical distribution of *S. longicauda* and

morphological variation among individuals that occur in sympatry in South Africa, we hypothesise the presence of hitherto unrecognised taxa.

This study aimed to characterise and delimit morphotypes of *S. longicauda*, with particular emphasis on sites where multiple, seemingly discrete, morphotypes co-exist. We therefore surveyed 14 sites where different morphotypes co-occur, to determine whether locally occurring morphotypes are morphologically and genetically discrete, thus potentially representing different taxa according to the Biological Species Concept. We then considered the broader distribution of morphotypes and sampled 36 sites representative of the geographical distribution and morphological variation of the species in South Africa, to characterise both morphological and genetic variation and establish phylogenetic relationships among morphotypes. We used this information to assess whether morphotypes may represent distinct taxa that can eventually be recognised as formal taxonomic units.

Material and Methods

Plant material sampling and populations surveyed

We aimed to include representatives across the observed phenotypic variation within *S. longicauda*, using the existing taxonomy as a starting point. *Satyrium longicauda* Lindl. var. *jacottetianum* (Kraenzl.) A. V. Hall. includes specimens that have spurs 13-26 mm long, and lateral sepals 4-7 mm long. The typical variety is based on the description of a specimen with deep-pink flowers and three leaves adpressed to the ground and was collected in Lesotho by Jacottet (Kraenzlin 1915). *Satyrium longicauda* var. *longicauda* Lindl. specimens have spurs 24-46 mm long, and laterals sepals 5-11 mm. *Satyrium longicauda* has a wide distribution and both varieties as recognised by Hall (1982) can be found in grasslands not far from sea level up to nearly 3100 m (Hall 1982; Johnson and Bytebier 2015; pers. obs.), indicating a considerable overlap and an unclear pattern in terms of habitat preferences of the varieties (Figure 3.1). Flowering spans a very long period, starting in the Austral winter through to autumn (August to April). On the other hand, *Satyrium rhodanthum* is confined to a small geographic region of KwaZulu-Natal at elevations from 750 to 950 m, and overlaps in range with *S. longicauda*. The species flowers from late October to December. In the present study, *S. rhodanthum* was included in the sampling, but *S. buchananii* Schltr., whose distribution is out of the South African territory could not be sampled for further measurements; however, the single accession used in the phylogenetic analysis of van der Niet and Linder (2008) was incorporated.

To characterise the morphological variation of *Satyrium longicauda* and *S. rhodanthum*, measurements were taken from plants that were collected during the flowering season from December 2012 until February 2020. Without excavating the root tuber, inflorescences and leaves were picked from sixty natural populations at thirty-six sites in the KwaZulu-Natal, Eastern Cape, Free State, Mpumalanga, and Limpopo provinces, representing part of the known geographical distribution and morphological variation of *S. longicauda* and *S. rhodanthum* in South Africa (Figure 3.1; Hall 1982; Johnson and Bytebier 2015).

Populations of *S. longicauda* usually comprise dozens to thousands of plants, so we subsampled by randomly collecting between 1-30 individuals per population depending on the availability of plants and stage of flowering. We selected individuals which were in full flower (including wilted and fresh flowers, and buds), and had leaves present. The final dataset comprised a total of 1730 individuals of *S. longicauda*, and 72 individuals of *S. rhodanthum* (sampled from three populations). In addition, three to six-leaf samples per population (or fewer depending on the number of individuals found) were collected and dried in silica gel for DNA extraction. Voucher specimens of all populations are deposited in the Bews Herbarium (NU) at the University of KwaZulu-Natal (Table S3.1). At 14 of the 36 sites, morphotypes occurred sympatrically (2-6 morphotypes per site). Although at many sympatric sites distinct morphotypes grow intermingled, in some cases there were microhabitat differences that resulted in spatial clustering among morphotypes. Nevertheless, these were considered sympatric because the distance between microhabitats was considered much smaller than the distance covered by pollinators and seed dispersal, suggesting that extensive gene flow was possible among morphotypes occurring in different microhabitats.

Identification of morphotypes

Based on initial analyses of morphological variants, we hypothesised the existence of eight morphotypes, that could be diagnosed based on unambiguous characters or character combinations, and that form the units of the downstream analyses (Figure 3.2). Once the groups were defined, we compared them against high-resolution digital images of the type specimens of *S. longicauda* (and *S. platystigma*), and *S. jacottetianum* available on JSTOR Global Plants (www.plants.jstor.org), and The United Herbaria of the University of Zurich and ETH (www.herbarien.uzh.ch), respectively. This comparison was done to evaluate which of the morphotypes matched each taxon concept. After observing the holotype of *S. longicauda* and *S.*

platystigma we noticed they seem to represent the same morphotype, thereby corroborating Hall's (1982) statement that the latter is a synonym of *S. longicauda*.

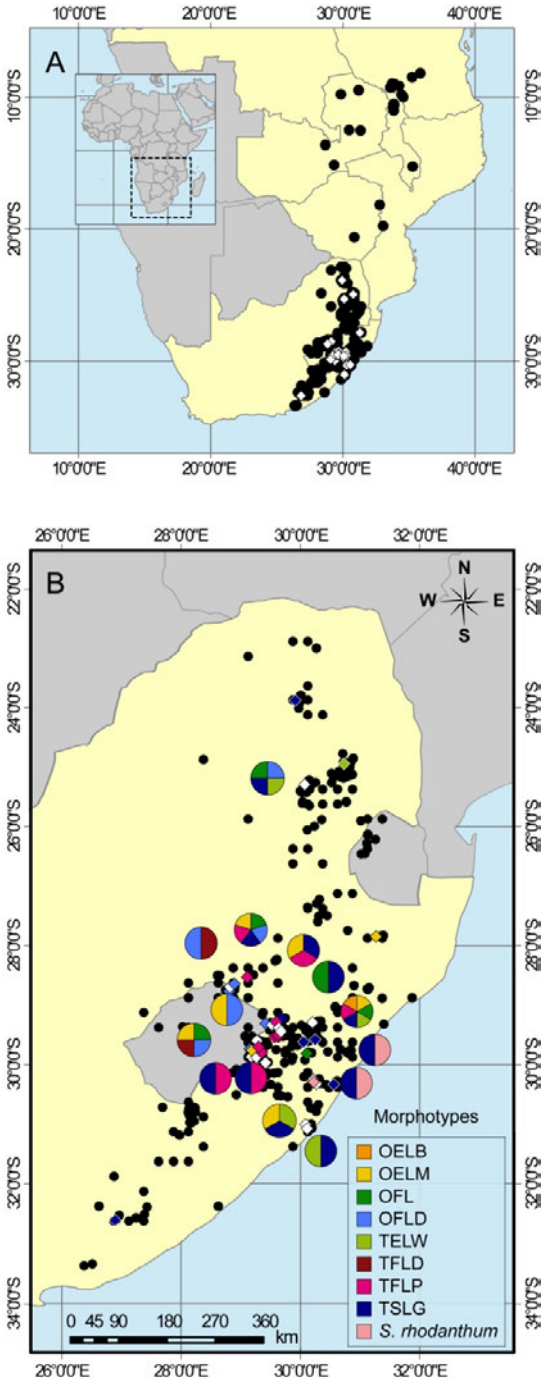
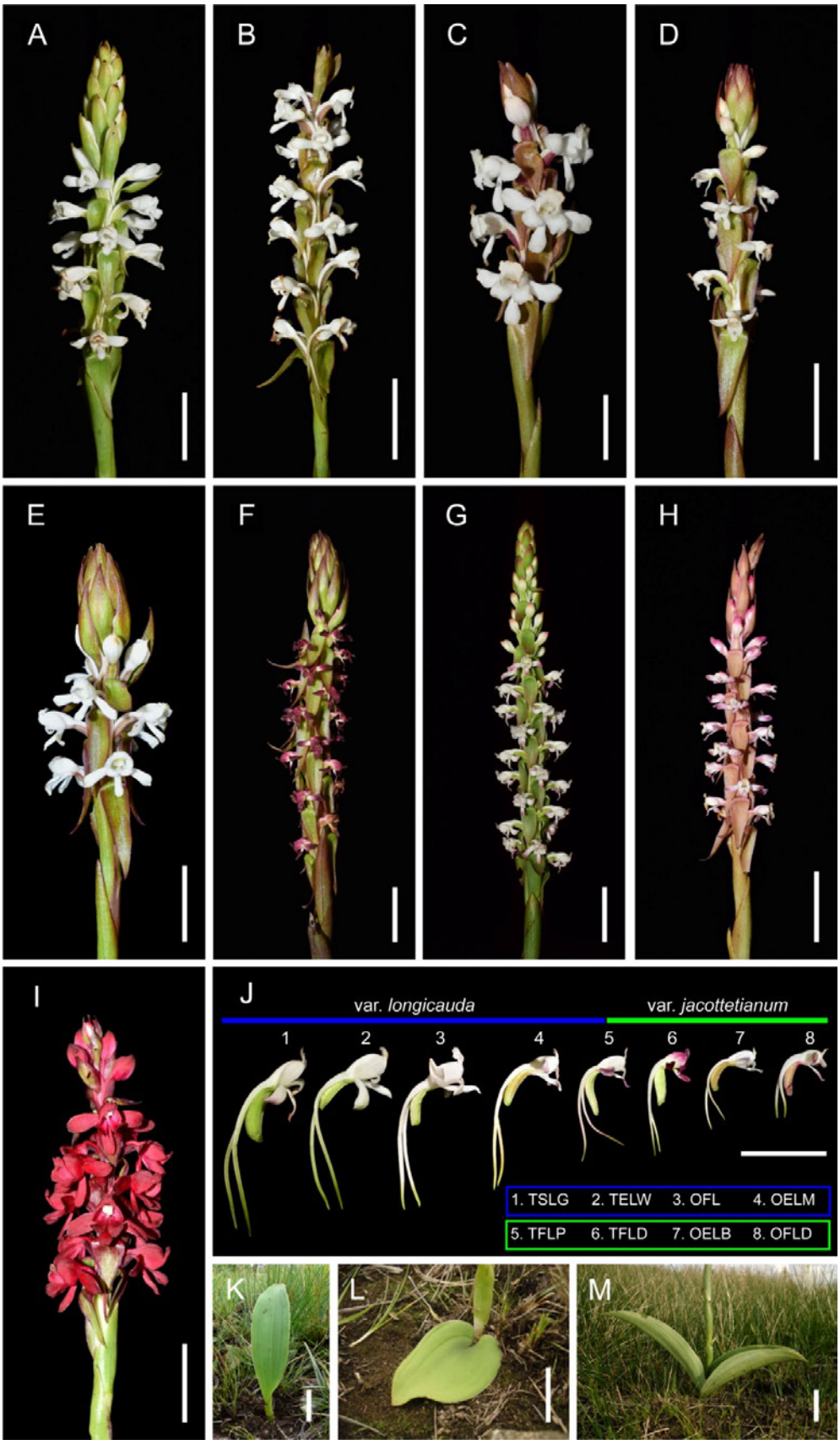


Figure 3.1. Geographical distribution of the *Satyrium longicauda* complex. A, Southern countries where the complex has been reported. B, in South Africa the *Satyrium longicauda* complex is distributed in the summer rainfall region. Black dots represent extant records based on herbaria specimens and sights in the field whereas diamonds show the 63 populations sampled for this study. Sympatric populations are indicated with white diamonds and these are linked with pie charts that represent the occurrence of morphotypes at each site.



(caption on next page)

Figure 3.2. Morphological variation of the *Satyrium longicauda* species complex. Inflorescences A-I. A, OELB = one erectl-bee (from MCZ-1671). B, OELM = one erect leaf-moth (from MCZ-1630). C, OFL = one flat leaf (from MCZ-1631). D, OFLD = one flat leaf-dwarf (from MCZ-1632). E, TELW = two erect leaves-wetland (from MCZ-1620). F, TFLD = two flat leaves-dwarf (from MCZ-1257). G, TSLG = two spreading leaves-giant (from MCZ-1704). H, TFLP = two flat leaves-pink (from MCZ-1664). I, *Satyrium rhodanthum* (from MCZ-1605). J, Comparison of flowers of both varieties of *S. longicauda* as delimitated by Hall (1982) based on spur length. Flower number 5 represents morphotype TFLP, which may be considered either variety. K, one erect leaf represented by an individual of the OELM morphotype. L, leaf of the OFL morphotype exemplifying the flat (adpressed to the ground) habit. M, distinctive pair of erect leaves of the TELW morphotype. Individuals which pictures were taken come from: A, E, G, K, L, and M, Mt Gilboa site; B, C, D and F represent populations from Tarn Cave; H, from Kamberg site, and *S. rhodanthum* (I) from Jolivet. Scale bars = 2 cm.

To distinguish the morphotypes within *S. longicauda*, we informally named them based on a combination of leaf characters (leaf number and position), plant size, flower colour, pollination data and habitat (Castañeda-Zárate *et al.* 2021; Chapter 2): one erect leaf-bee (OELB), one erect leaf-moth (OELM), one flat leaf (OFL), one flat leaf-dwarf (OFLD), two erect leaves-wetland (TELW), two flat leaves-dwarf (TFLD), two flat leaves-pink (TFLP), and two spreading leaves-giant (TSLG). Any reference to the term "morphotypes" below also includes *S. rhodanthum*.

Morphological character selection and measurement, and data pre-processing

To quantify and evaluate whether the variation observed in the morphotypes identified by visual evaluation conforms to discrete groups in multivariate morphospace, we measured, counted, and recorded a combination of fifteen floral and vegetative characters of the sampled individuals. These characters were selected because they capture the variation in reproductive and vegetative morphology. For floral measurements, we used a randomly chosen single and unpollinated flower (as inferred by the presence of both viscidia and absence of massulae on the stigma) from the middle segment of the inflorescence, whereas the longest leaf (for morphotypes with multiple leaves) was used to obtain measurements. Quantitative characters included: plant height, inflorescence length, stem base diameter, inflorescence stem diameter, leaf length and width, galea aperture height, galea margin height, lateral sepal length, spur length (considered as the distance between the spur tip and the site of fusion with the galea), and functional spur length (including the distance between spur fusion and viscidium). The number of both flowers (including buds) and leaves were included too. Two qualitative characters including the position of the leaves (erect versus adpressed to the ground to prostrate) and flower colour (white versus pink to red) were also recorded. Plant height, inflorescence length, and leaf length and width were measured with a tailor tape to the nearest 1 mm and other continuous characters were measured with a pair of digital

calipers to the nearest 0.01 mm. Qualitative traits and the number of leaves were coded as binary variables.

Missing data accounted for c. 2% of all measurements. To prepare the data for statistical analyses, missing values were replaced with character means using the function *imputePCA* of the missMDA R package (Josse and Husson 2016). Spur length (from where spurs split to the tip of the spurs) and functional spur length (rostellum to tip of each spur) were highly correlated ($r^2 = 0.98$). Therefore, we decided to remove the latter (functional spur length) for subsequent analyses because we considered spur length more informative from a taxonomic perspective as this can be measured on herbarium specimens without the need to dissect flowers. All statistical analyses were carried out using R v. 3.6.3 (R Core Team 2020).

Assessment of morphological variation

Analysis of variation at a local scale (sympatric populations)

To investigate the extent to which morphotypes that co-occur comprise discrete entities (and therefore may represent separate species under the Biological Species Concept (BSC)), we implemented Canonical Variate Analyses (CVA) and Finite Gaussian Mixture Models (FGMM). Both analyses were implemented for each of the 14 sympatric sites.

We first performed CVAs followed by a Multivariate Analysis of Variance (MANOVA) and a Classificatory Discriminant Analysis (CDA) using *a priori* assignment of individuals to any of the morphotypes based on visual inspection. This analysis tests whether characters that can be used by non-specialists for identification support the classification of morphotypes. For these analyses, we used standardised quantitative characters. The CVA was carried out to identify the best predictor characters underlying the separation of *a priori* identified morphotypes using the *lda* function of the MASS package (Venables and Ripley 2002). A MANOVA was subsequently applied to test if the means of all morphotypes differ using Wilks' Lambda (Λ) as a test statistic (Gotelli and Ellison 2013). Finally, a CDA was conducted to corroborate the classification of the individuals assigned initially to each morphotype. Because CVA summarises 100% variance in one canonical function for sites with only two morphotypes that cannot be represented in a scatterplot, we used Principal Component Analysis (PCA) based on a correlation matrix to visualise how each morphotype is distributed in morphospace for each site.

To assess whether patterns of variation based on the set of all characters can be confirmed by discontinuity in values for a small subset of key characters, we also performed bivariate and univariate analyses. Bivariate analyses consisted of scatterplots to visualise the relationship between spur and lateral sepal length. These characters were selected as they were among the more informative characters for separating morphotypes in the multivariate analyses and because they were used by Hall (1982) to distinguish among varieties of *S. longicauda* and *S. buchananii* (Hall 1982). For univariate analyses we focused on spur length, which is a key taxonomic character above and below species-level in *Satyrium* (Johnson 1997; Johnson and Kurzweil 1998). Finite Gaussian Mixture Models (FGMM) on spur length was used to estimate the number of components present in the data set using the *densityMclust* function of the mclust package (Scrucca *et al.* 2016). The number of components obtained may indicate the minimum number of diagnosable groups (e.g. gene pools) at each site. This approach computes the Bayesian Information Criterion (BIC) to assess the number of clusters in the data. Finite mixture models provide a flexible semi-parametric model-based method to estimate an unknown density function (Scrucca *et al.* 2016; McLachlan *et al.* 2019). Density estimation was also obtained for sites with only a single morphotype present, to evaluate whether a single component was recovered in contrast with sympatric sites for which multiple components were expected. In all cases, we only included morphotypes for which at least 15 individuals were measured. This arbitrary cut-off was chosen to avoid retrieving multiple components as an artefact of undersampling.

Morphological variation at a regional scale

Morphotypes were represented by 1-17 populations (Table S3.1). The number of putative populations of each morphotype in ascending order was OELB = 1, TFLD = 2, *S. rhodanthum* = 3, TELW = 5, OFL = 6, OELM = 8, OFLD = 9, TFLP = 12, and TSLG = 17 (mean ± SD = 7 ± 5.15). To test the hypothesis that the morphotypes initially identified in the preliminary assessment represent distinguishable groups across their distribution, a discriminant analysis for 1802 individuals was performed in the same way as described above. To assess whether different populations of the same morphotype cluster together we performed a Hierarchical Cluster Analysis (HCA) using a combined dataset including both the quantitative and qualitative characters. First a morphological distance matrix was calculated based on the population mean values of each character. Gower distance (Gower 1971) on unweighted variables was employed to calculate the dissimilarity matrix because it allows the combination of numeric and non-numeric variables, using the *daisy* function from the

cluster package (Maechler *et al.* 2016). Operational Taxonomic Units (OTUs) were then joined together implementing the Unweighted Pair-Group Method using Arithmetic Averages (UPGMA) as a clustering algorithm with the R function *hclust* from the fastcluster package (Müllner 2013). The goodness of fit was measured by calculating the co-phenetic correlation coefficient (r) using the distance matrix (Gower distance) and the distances obtained in the dendrogram (Sokal and Sneath 1963; Lessig 1972).

Assessment of genetic variation

Molecular methods

To establish whether morphotypes that occur in sympatry are genetically distinct, to infer phylogenetic relationships among morphotypes, and to test the monophyly of *S. longicauda*, representatives from thirty six putative populations at 20 sites were sampled (Table S3.1). We aimed to generate sequences from at least three of the six individuals collected per population. However, for a few populations only one or two sequences could be obtained due to occasional PCR amplification or sequencing failure, resulting in a total of one hundred and eighteen accessions (Table S3.1).

DNA extraction was performed with DNeasy® Plant Mini Kits (Qiagen, Hilden, Germany) from material dried on silica gel following the manufacturer´s protocol. The nuclear ribosomal internal transcribed spacer (nrITS) region was amplified using the ITS5 and ITS4 primers (White *et al.* 1990), or the AB101 and AB102 primers (Sun *et al.* 1994; Douzery *et al.* 1999). The polymerase chain reaction was performed in a total volume of 25 µl. The PCR mix for reactions done with the ITS4 and ITS5 primers consisted of 12.5 µL of OneTaq® Quick-Load® Master Mix with Standard Buffer (New England Biolabs), 1 µL bovine serum albumin (10 ng/µL), 0.5 µL each primer (10 µM), 10.5 µL distilled H_2O, and 1 µL of the extracted DNA. Amplification was carried out in a Veriti™ 96-Well Thermal Cycler. The cycling protocol started with an initial 30 sec of denaturation at 94°C, followed by 35 cycles of 30 sec denaturation at 94°C, 1 min annealing at 53°C, 1 min extension at 68°C, and was finished with a final extension of 5 min at 68°C. PCR products were purified and sequenced with the same primers as were used for the PCR amplification at the Central Analytic Facilities (CAF) of Stellenbosch University (South Africa). For samples amplified using AB101 and AB102 primers we followed the protocol described in van der Niet *et al.* (2005). PCR products were outsourced for purification and sequencing to Macrogen (Macrogen Europe, Amsterdam, The Netherlands).

Twelve accessions showed extensive site (but not length) polymorphism, resulting in the presence of ambiguous base calls in the chromatograms. These were cloned using a TOPO® TA Cloning® Kit and One Shot® TOP10 chemically competent cells (Invitrogen, Carlsbad, California) following the manufacturer's protocol. For each accession, eight colonies were selected and amplified using the specifications described above for the ITS4 and ITS5 primer, but with an additional final cycle of 15 min at 72°C as suggested by the manufacturer. Chromatograms were assembled in Geneious® 10.2.2 (Kearse *et al.* 2012). A comparison of the sequences of the cloned PCR products against a reference sequence revealed the presence of chimeric sequences (Hugenholtz and Huber 2003; Haas *et al.* 2011). In the absence of information on whether these represent PCR artefacts or real alleles, we decided to exclude these sequences from the matrix, except for samples of the TSLG morphotype from Coleford, which showed no evidence of chimeric sequences. Moreover, two cloned sequences of the same morphotype from Verloren Valei obtained by van der Niet and Linder (2008) available on GenBank were also included in the matrix.

Ambiguous base calls in consensus sequences were coded with the IUPAC polymorphic coding. The alignment was performed in the MAFFT (v7.471) online service (Katoh *et al.* 2019) using the default parameters of the FFT-NS-1 method. Indels in the alignment were coded as present or absent (Simmons and Ochoterena 2000) using the program FastGap 1.2 (Borchsenius 2009) and added as additional characters to the matrix.

Haplotype analysis

Given that morphological variants may represent different cohorts of a panmictic population, we tested whether distinct morphotypes within sites are also genetically distinct, by constructing haplotype networks under a median-joining inference method (Bandelt *et al.* 1999) using POPART 1.7 (Leigh and Bryant 2015). These analyses were done separately for each sympatric site for which accessions from multiple morphotypes were available, by omitting all remaining sequences from the aligned matrix. We also performed a haplotype network analysis on all sequences to test whether morphotypes conform to different haplotypes.

Phylogenetic analysis

To assess the monophyly of *S. longicauda*, the two varieties, and each of the morphotypes, and to reconstruct phylogenetic relationships among the morphotypes, phylogenetic analyses were performed through maximum parsimony (Farris 1970; Fitch 1971) and Bayesian inference (Huelsenbeck and Ronquist 2001; Ronquist *et al.* 2012; Nascimento *et al.* 2017). For these analyses ten additional sequences including one accession of *S. buchananii*, which was nested within *S. longicauda* in previous studies (van der Niet and Linder 2008; van der Niet *et al.* 2011), one of *S. monadenum* Schltr., three of *S. sceptrum* Schltr., and four of *S. neglectum* Schltr. were sourced from GenBank. These taxa comprise the closest relatives of *S. longicauda* (van der Niet and Linder 2008; van der Niet 2017). *Satyrium bicorne* (L.) Thunb. was added for the purpose of rooting the tree (Table S3.1). The aligned matrix, including coded gaps for 130 ITS sequences, was used as input data.

Parsimony analyses were done using a heuristic search comprising 10,000 starting trees (Wagner trees) and retaining 100 trees per replicate followed by tree bisection-reconnection (TBR) branch swapping. Resulting trees from the initial search saved in memory were submitted to a new round of TBR branch swapping to find additional equally parsimonious trees. Bootstrap resampling (Felsenstein 1985) was used to evaluate the support of the nodes of the strict consensus tree, based on 1,000 replicates and summarised using absolute frequencies. All characters were treated as unordered and equally weighted. This analysis was done using TNT 1.5 (Goloboff *et al.* 2008; Goloboff and Catalano 2016).

Bayesian inference analysis was performed in MrBayes v.3.2.7a (Ronquist *et al.* 2012) using the CIPRES Science Gateway 3.3 (Miller *et al.* 2015) implementing the method (including choice of models of DNA sequence evolution) described in van der Niet and Linder (2008). Gap evolution was modelled using a Markov model (Lewis 2001). The analysis was carried out for five million generations, sampling trees and parameters every 1,000 generations and discarding 1,000 initial samples (20%) as burnin.

Comparison of morphological and genetic distances

To investigate whether morphological and genetic distances are correlated, we used a Mantel test (Mantel 1967). Such correlation is expected if morphological divergence evolves gradually through time, and distinct morphological units can then be considered good proxies for incipient lineages. A

morphological distance (dissimilarity) matrix was calculated based on population mean values of each variable. We calculated morphological distance using Gower distances (Gower 1971) on the morphological dataset of thirty-six populations for which ITS sequences were obtained. The genetic distances for the same populations were measured as uncorrected (p) distance using the function *dist.hamming* from the package phangorn (Schliep 2011), using a single randomly selected sequence per population. To generate a null distribution, 10,000 permutations of the matrices were performed using the function *mantel.rtest* as implemented in the package ade4 (Chessel *et al.* 2004; Dray and Dufour 2007; Dray *et al.* 2007).

Results

Morphological and genetic variation at the local scale

Of the 63 putative populations of morphotypes sampled, 41 (65.1%) were found to occurr together in sympatry with at least one other morphotype (Table S3.1; Figure 3.1). In total, 12 different combinations of two up to six different morphotypes were observed in sympatry (Figure 3.1; Table 3.1). The mean number (± SD) of morphotypes at sympatric sites was 2.93 ± 1.33. Overall, morphotypes occurring in sympatry were recovered as morphologically different from each other based on multivariate analyses of the eleven characters (all cases: $p < 0.001$; Table 3.1; Figure 3.3). In all cases, spur length was among the most important characters for morphotype discrimination (Figure 3.2J; Figure 3.3). Multivariate analyses, particularly for sites with more than three sympatric morphotypes such as Mt Gilboa and Tarn Cave, showed partial overlap among morphotypes. In spite of this, classificatory analyses showed high percentages of correct *a posteriori* assignment ranging from 83.33% to 100% (Table 3.1).

Scatterplots of lateral sepal length and spur length revealed clear discontinuities between morphotypes (Figure 3.4). Similar to the multivariate analyses, discontinuous variation was more evident at sites where only two morphotypes were present, although not exclusively so since clear differences in the scatterplot analysis were present among the four morphotypes at Tarn Cave (Figure 3.4). At other sites where three or more morphotypes co-existed the correlation between lateral sepal length and spur length showed recurrent overlap.

Univariate analyses of spur length based on a subsample of 34 sympatric populations representing twelve sites, revealed the presence of two components for most sympatric sites. The exception was

Bushman's Nek for which four components were recovered but for which only identified two morphotypes were identified. Likewise, Sentinel Peak and Kamberg for which only one component was obtained, two and three morphologically different morphotypes occur respectively (Figure 3.5). The number of components for eleven sites characterised by a single morphotype was one, except for Garden Castle where a second component was retrieved.

Assessment of the genetic variation showed that sympatric morphologically distinct morphotypes are also genetically distinct, as reflected by the frequent presence of unique haplotypes characterizing each morphotype (Figure 3.6). The only exception was observed for TSLG and TFLP, which are represented by a single haplotype at sites where they co-occur.

Morphotypes discrimination at the regional scale

CVA successfully distinguished all morphotypes in eight statistically significant canonical variates (ΛWilks = 0.0092, F = 201.39, *d.f.* = 8, $P < 0.001$. Approximately 90% of the variance was summarised in the three first canonical variates (Table 3.2, Figure 3.7A). The first canonical variate explained 49.16% of the variance. Spur length and galea aperture height were characters with high loadings (Table 3.2). The second canonical variate explained 29.63% of the variance, with spur length, lateral sepal length, and galea aperture height as the most significant characters accounting for high loadings (Table 3.2). Leaf length and width, and galea margin height were the most discriminating characters for morphotype delimitation in the third canonical variate, explaining 10.34% of the variance (Table 3.2).

Although statistically significant, the remaining five canonical variates together explained only an additional 10.87% of the variance (Table 3.2). CDA correctly assigned 85.63% of the specimens into their respective groups defined *a priori* (Table 3.3). Overall, high values of correct *a posteriori* classification between 76.81% and 94.86% were observed with the exception of the TFLD morphotype with 54.29% of misclassification (Table 3.3). Of this morphotype, 37.14% of individuals were reclassified into the TFLP morphotype, whereas 17.17% of individuals were reclassified into OFLD morphotypes. For *S. rhodanthum* 86.11% of individuals were correctly classified (Table 3.3).

Table 3.1. Confusion table comparing the original group assignment of the morphotypes of sympatric populations of the *Satyrium longicauda* complex to the assignment made from the classification functions. For each site, ANOVA or MANOVA showed significantly different mean values between populations (in all cases, *** = $p < 0.001$). Correct classification values are given as percentages.

Site/Morphotype	Classification values (%)									ΛWilks	*F*	df	Significance
	OELB	OELM	OFL	OFLD	TELW	TFLD	TFLP	TSLG	*S.rhodanthum*				
Bushman's Nek							100	96.55		—	565.5	1	***
Coleford							100	100		—	755.07	1	***
Highflats								100	100	—	1210	1	***
Highmoor		100	100	100			100	96.77		0.008	33.56	4	***
Jolivet								100	100	—	1357	1	***
Kamberg		94.59					85.71	93.33		0.077	146.21	2	***
Mpofana Rd			100					100		—	138.9	1	***
Mt Gilboa	98.33	98.85	97.89		92.45		92	97.43		0.004	218.1	5	***
Red Desert					100		100	100		—	463.1	1	***
Sani Pass		100		100						—	877.9	1	***
Sentinel Peak				100		100				—	475.4	1	***
Tarn Cave		94.44	100	100		90.70				0.007	326.78	3	***
Umtamvuna		83.33			93.33			100		0.067	165.32	2	***
Verloren Valei			96.96	100	100			92.30		0.009	126.57	3	***

Table 3.2. Results of Canonical Variate Analysis obtained using 11 quantitative characters of the *Satyrium longicauda* complex. Loadings with higher contributions are in bold.

	Canonical Variate		
Character	1	2	3
A. Plant height	0.0877	0.1928	-0.2895
B. Inflorescence length	-0.1324	-0.2697	-0.0900
C. Number of flowers	0.0192	-0.5258	0.1493
D. Stem diameter	0.1383	-0.1331	0.2270
E. Inflorescence diameter	-0.0866	-0.0895	0.1983
F. Largest leaf length	0.3478	0.3590	**1.1410**
G. Leaf width	-0.0146	-0.4812	**-0.7644**
H. Galea aperture height	**0.8045**	**0.6298**	0.4016
I. Galea margin height	-0.0386	0.1331	**-0.6246**
J. Lateral sepal length	0.2020	**0.8104**	-0.4653
K. Spur length	**1.3301**	**-1.1922**	-0.2798
Proportion of variance	0.4916	0.2963	0.1034

Table 3.3. Confusion table comparing the original group assignment of the morphotypes of the *Satyrium longicauda* complex to the assignment made from the classification functions.

Observed group	Assigned to group								
(N)	OELB	OELM	OFL	OFLD	TELW	TFLD	TSLG	TFLP	S. rho
OELB (60)	53	0	0	0	0	0	0	0	0
OELM (276)	0	212	6	9	11	0	16	14	0
OFL (190)	0	10	154	0	6	0	12	2	0
OFLD (214)	5	13	0	203	0	12	0	12	0
TELW (158)	0	24	0	0	133	0	8	0	10
TFLD (70)	2	2	0	1	0	32	0	5	0
TSLG (488)	0	3	0	0	6	0	430	5	0
TFLP(274)	0	11	28	1	0	26	22	236	0
S. rho (72)	0	1	2	0	2	0	0	0	62
Correct classification (%)	88.33	76.81	81.05	94.86	84.18	45.71	88.11	86.13	86.11

The HCA showed a relatively high coefficient of cophenetic correlation (r = 0.77) indicating a good fit between the distance matrix and the dendrogram. The dendrogram resulted in two main aggregations (at 3.5 Gower Distance) comprising exclusively of populations of morphotypes with one and two leaves, respectively (Figure 3.7B). The unifoliate group (Group I) included two subclusters. The first subcluster included 1) OELB nested within OELM, 2) OFL, 3) OFLD, whereas the second subcluster only included populations of *S. rhodanthum*. Morphotypes within the unifoliate group were distinguished for their overall small size (e.g. plant height = 282.26 mm; lateral sepal length = 8.08 mm; spur length = 24.67 mm, median values). Within the bifoliate group, three main subclusters were obtained. The first subcluster included populations of the TSLG morphotype. The

second subcluster recovered only populations of the TELW morphotype. The third subcluster grouped populations of the TFLD and TFLP morphotypes. Overall, populations within the bifoliate cluster (Group II) include individuals with a median height = 404.24 mm, median lateral sepal length = 8.62 mm, and median spur length = 34.38 mm.

Excluding clonal sequences, we detected fifteen unique haplotypes (Figure 3.8). One haplotype was shared between populations of TFLD, TSLG, and TFLP (Figure 3.8). Morphotypes represented by several haplotypes included OFLD with five haplotypes followed by OELM with four, and TELW and TSLG with two haplotypes each (Figure 3.8). OFLD from Witsieshoek and OELM from Mt Gilboa were polymorphic. Although all four populations of OFL were found to share a single haplotype, it is not exclusive as it is shared with two populations of OFLD (Figure 3.8). OELB and *S. rhodanthum* each had their own unique haplotype (Figure 3.8).

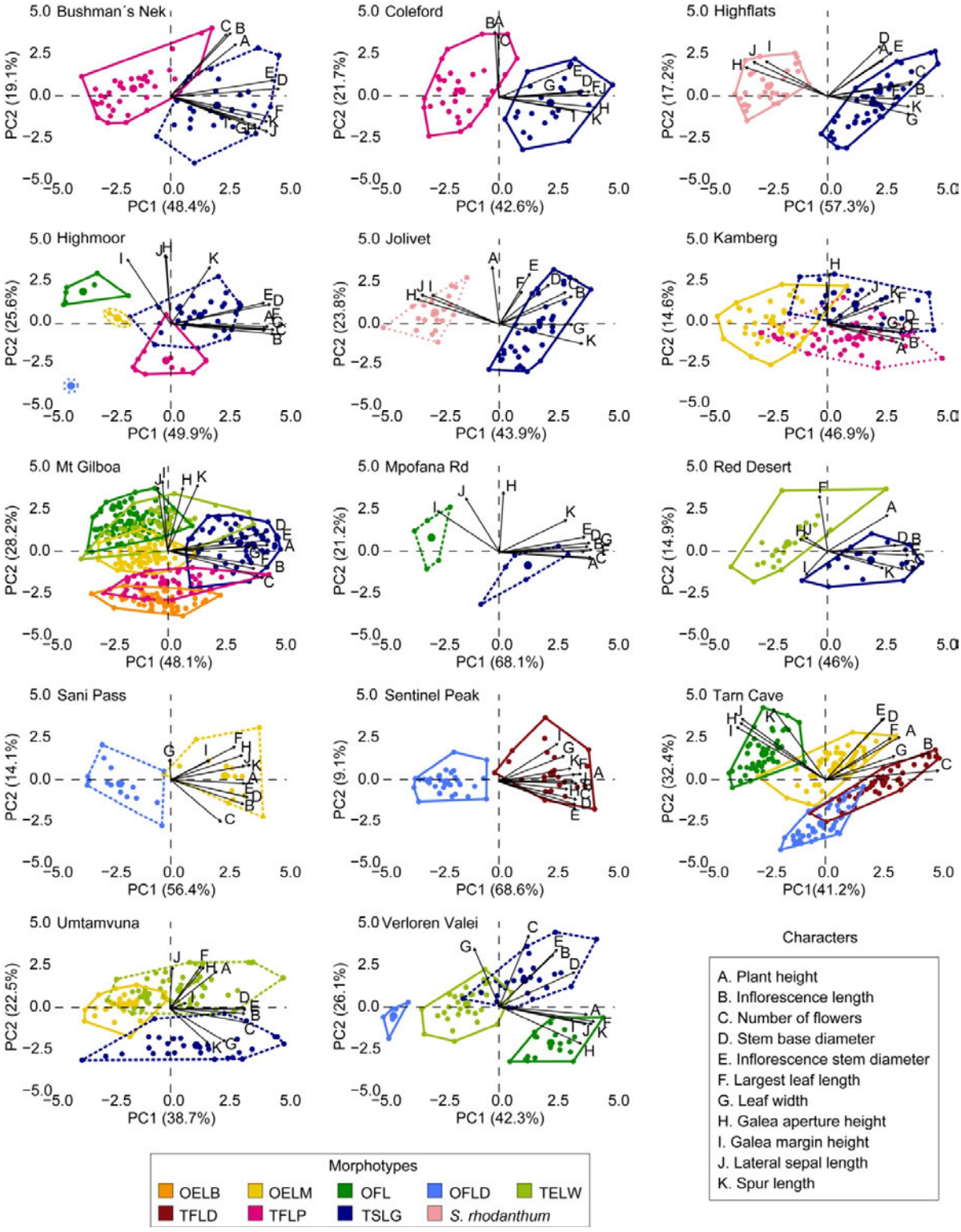


Figure 3.3. First two Principal Components (PC) based on analysis of eleven morphological characters. Gray arrows illustrate the influence of the eigenvectors on the principal components. Individuals of each population are enclosed in convex hulls. Convex hulls with solid line represent populations for which sequences were obtained whereas dashed convex hulls indicate populations not present in the phylogenetic analysis. Percentages of variance explained per each PC are given in parentheses.

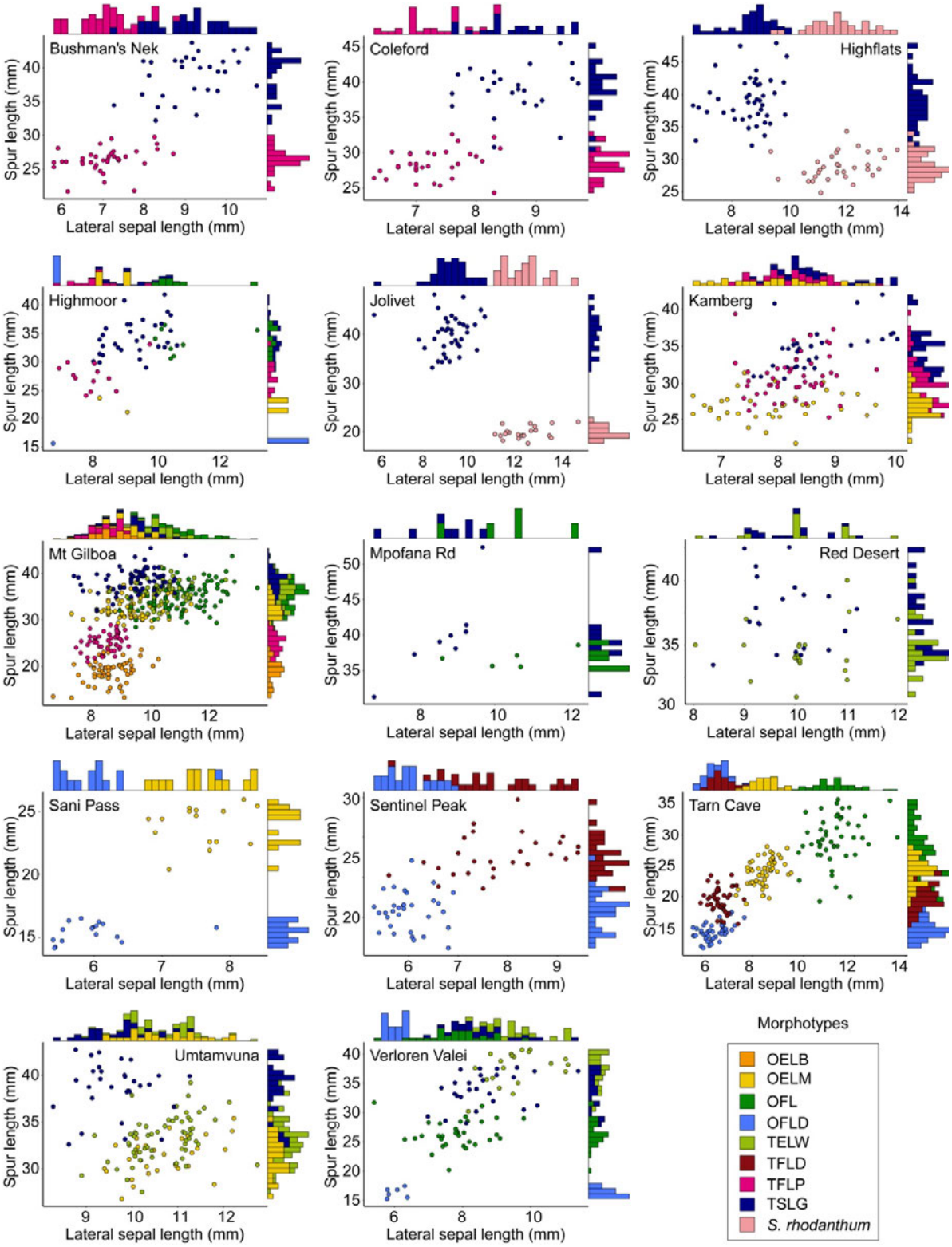


Figure 3.4. Pairwise scatterplots between lateral sepal length and spur length for the sympatric sites. Histograms show frequency distributions.

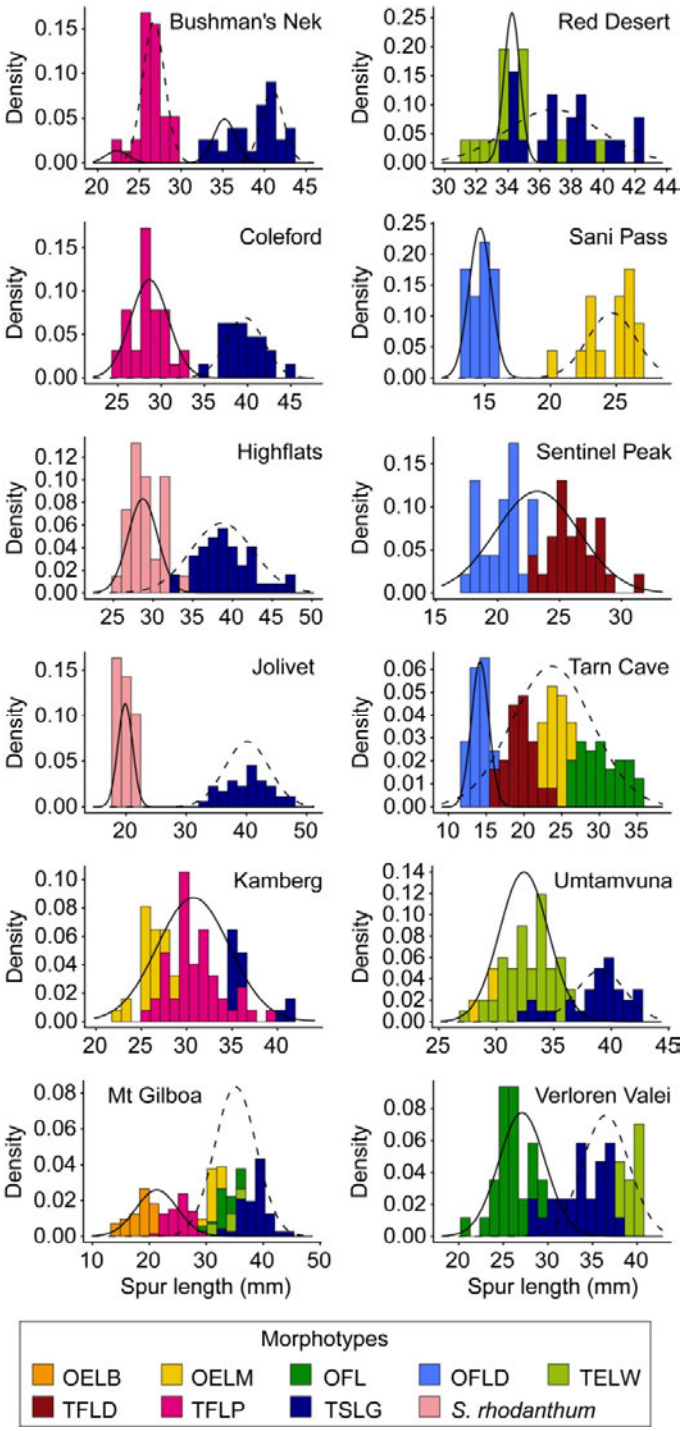


Figure 3.5. Density estimation plots based on spur length. Each Gaussian distribution is indicated with either with a solid or dashed line.

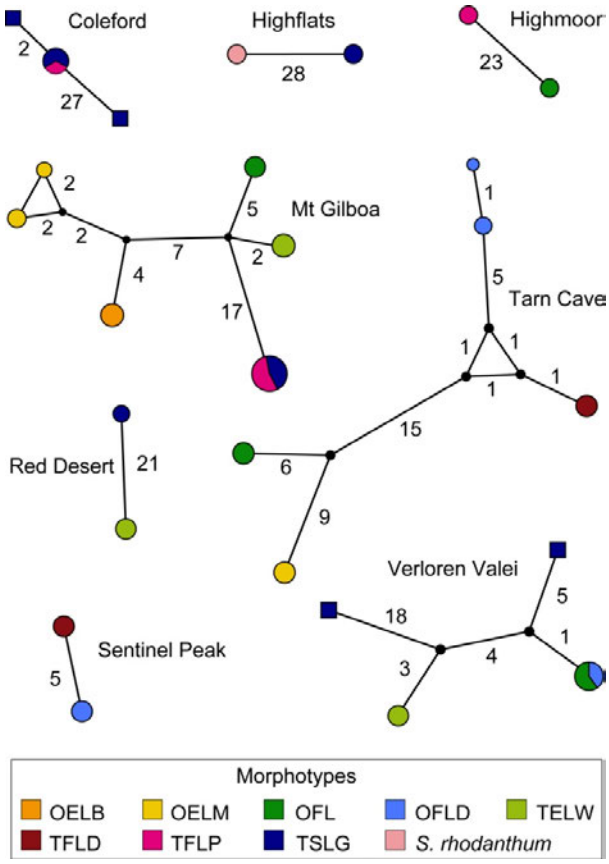


Figure 3.6. Median-joining haplotype network based on nuclear ITS sequences. Numbers of mutations are indicated next to the lines between haplotypes. Hypothetical unsampled haplotypes are indicated with black circles. The squares represent cloned sequences.

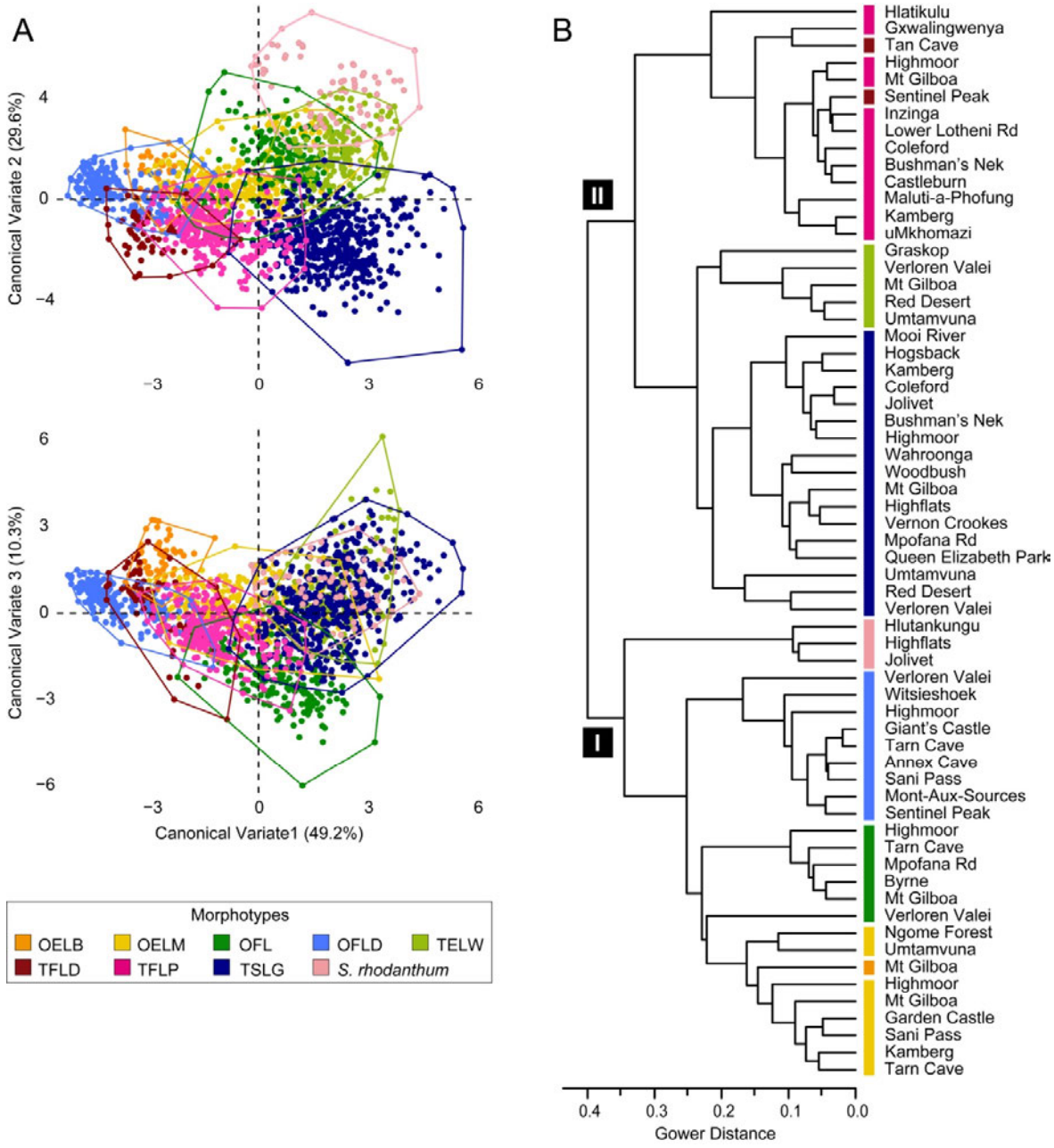


Figure 3.7. Morphological and genetic variation of the *S. longicauda* complex at regional scale. A, Canonical Variate Analysis (CVA) based on eleven morphological characters. The first three axes explained 49.2%, 29.6% and 10.3% of the total variance, respectively. B, Dendrogram representing 63 populations of the *Satyrium longicauda* complex showing the clusters recovered using Gower Distances and the UPGMA clustering algorithm. Group I comprises morphotypes characterised for the presence of one leaf and group II comprises all morphotypes with two leaves. Cophenetic correlation coefficient = 0.77.

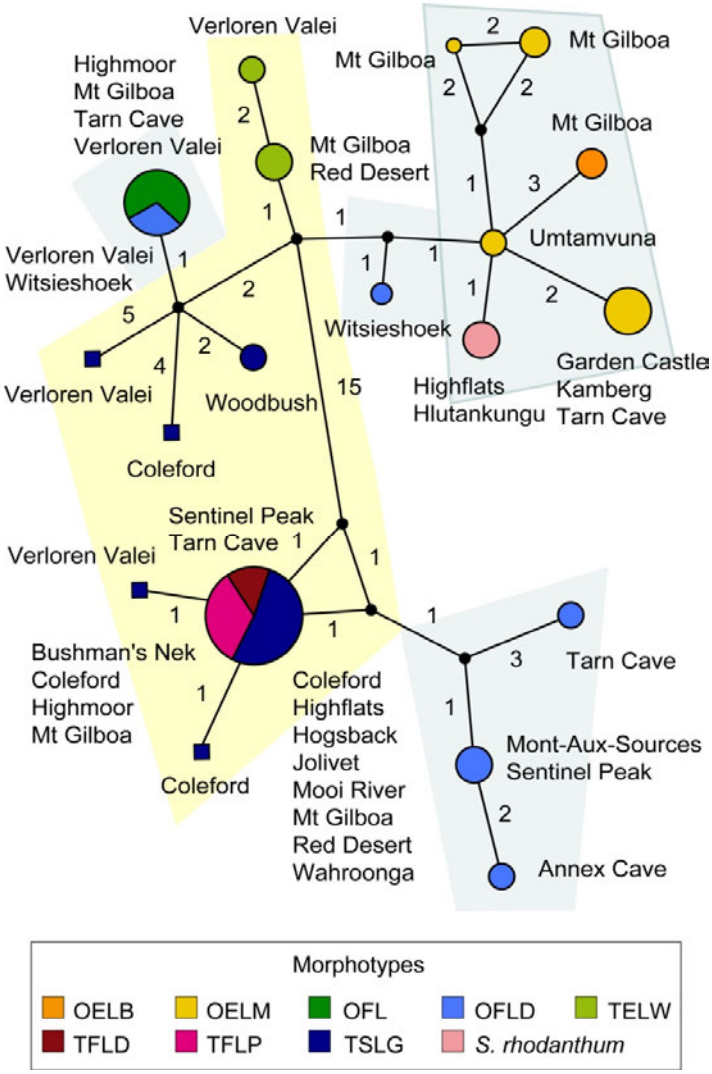


Figure 3.8. Median-Joining haplotype network of 36 populations for which DNA sequences were obtained. Numbers represent mutations. Locality names are given next to each representative haplotype. Hypothetical haplotypes are indicated with black circles. The squares represent cloned sequences. Morphotype colour code is indicated in the legend. Gray polygons represent the unifoliate group. Gray polygon with bolded edge includes morphotypes that bear one erect leaf. The yellow polygon encloses the bifoliate group.

Phylogenetic Analyses

Parsimony and Bayesian phylogenetic analyses recovered congruent topologies, but given the higher statistical support in the Bayesian analysis, it is displayed here and used for discussion (Figure 3.9). Two major clades (A and B) were recovered, both with posterior probability (PP) of 1.00. Within clade A, a clade comprising the accessions of *S. rhodanthum* is embedded within a subclade that otherwise contains a monophyletic OELB, a paraphyletic OELM, and two accessions of OFLD (PP

1.00). A second subclade with strong support (PP 1.00) comprises further accessions of OFLD, as well as all accessions of OFL and a cloned accession of TSLG. Sister to this, was a clade with TSLG from Woodbush and another cloned accession of TSLG. Accessions of TELW comprise a strongly supported monophyletic clade (PP 0.98). Clade A also includes *S. buchananii*. Clade B is less well resolved. It includes a polytomy with all accessions of TFLD and TFLP and some accessions of TSLG (PP 1.00). Further accessions of TSLG are unresolved, whereas a clade of OFLD received PP of 1.0 as part of Clade B. These results show that *S. longicauda* is not monophyletic. Unlike the HCA, the phylogenetic tree did not provide evidence for consistent morphotypes monophyly of morphotypes, or grouping by leaf number (Figure 3.9). The morphotypes assigned to the two currently recognised varieties of *S. longicauda* are also not retrieved as reciprocally monophyletic clades (Figure 3.9).

The Mantel test revealed that there was a positive and significant correlation between morphological and genetic distances ($r = 0.25$; $p < 0.001$; Figure 3.10).

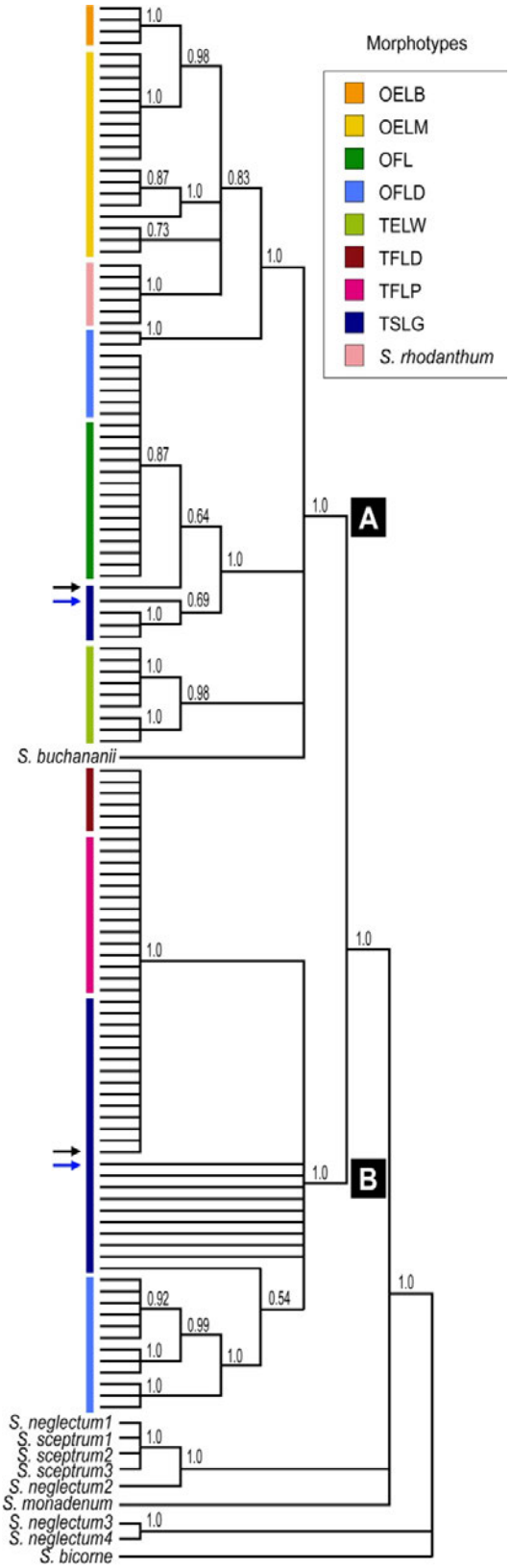


Figure 3.9. Phylogenetic relationships based on Bayesian Inference of nuclear DNA (ITS). Numbers above nodes represents Bayesian posterior probabilities. Arrows indicate cloned sequences of TSLG from Coleford (black) and Verloren Valei (blue). Black squares show the two main clades recovered.

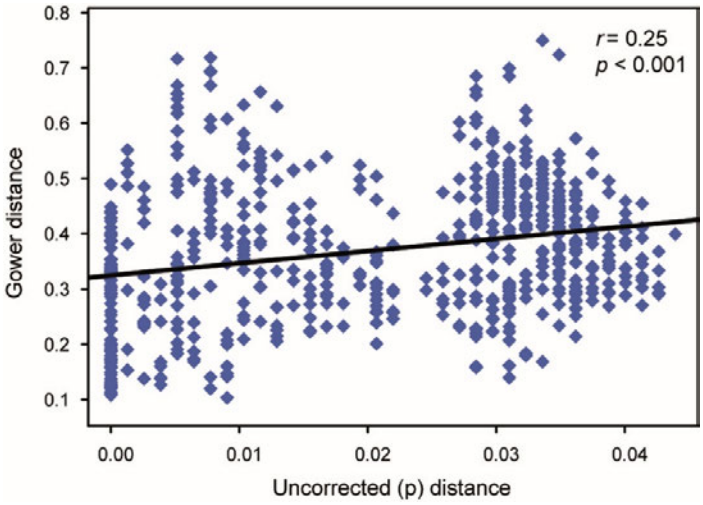


Figure 3.10. Mantel test for correlation between genetic (uncorrected (p) distance) and morphological (Gower distance) distances based on 1,000 random permutations.

Discussion

The analyses of the variation of the *S. longicauda* complex across a large part of its range represent a first attempt to characterise the variation from both a combined morphological and genetic perspective. The approach based on population-level sampling of fresh material provides evidence that supports Hall's (1982) statement about the presence of multiple taxa within *S. longicauda*. However, rather than two, we found evidence for at least eight discrete units that can be reliably identified by using a combination of floral and vegetative characters (Table 3.1 to 3.3; Figure 3.2, 3.7-3.9). These morphotypes are distinct entities in sympatry, a scenario often observed in this species complex, based on multivariate, bivariate and univariate data (Table 3.1; Figure 3.3 to 3.6). The genetic differentiation of some of the morphotypes suggests that intraspecific morphological variation might reflect early stages of divergence (Figure 3.8). Morphotypes are not only distinct in sympatry, but also at a regional scale, possibly reflecting their status as evolutionary lineages, which was partly supported by haplotype and phylogenetic analyses. *Satyrium rhodanthum* and *S. buchananii* are both phylogenetically nested inside *S. longicauda* (Figure 3.9; van der Niet and Linder 2008; van der Niet *et al.* 2011; van der Niet 2017), suggesting profound morphological evolution within the species complex.

Variation at the local scale

According to the Biological Species Concept, plant populations of taxa that maintain their integrity in sympatry could be considered different species (Mayr 1992, 2000). Several diagnostic tools are available to test this theoretical concept. In particular the presence of non-overlapping variation in morphological characters, and distinct genetic signatures are consistent with the presence of multiple evolutionary lineages. Principal Component and Discriminant Analysis of eleven morphological characters successfully distinguished most morphotypes at sympatric sites (Figure 3.3). Although some overlap in the distribution of certain taxa was observed at some sites (Figure 3.3), high values of *a posteriori* classification reinforce the hypothesis about the coexistence of multiple taxa (Table 3.1). Additionally, bivariate analysis of lateral sepal and spur length supported recognition of multiple morphotypes at several sympatric sites (Figure 3.4), thereby also supporting the existence of multiple taxa (Hall 1982). Finally, Gaussian Finite Mixture Analysis of spur length recovered the presence of at least two components at most sites. Indeed, spur length was previously used by Hall (1982) to diagnose the two varieties (Figure 3.2J). For sites with more than two morphotypes present (Mt Gilboa, 6 morphotypes; Tarn Cave, 4 morphotypes), the univariate analysis underestimated the number of morphotypes, as opposed to the results generated with multivariate analyses and scatterplots. This suggests that a single morphological character may be inadequate for taxon delimitation. Although some inconsistencies in the number of morphotypes identified by multi-, bi-, and univariate analyses exist, they all confirmed the presence of multiple morphotypes at sympatric sites.

Median-joining haplotype networks detected private ITS haplotypes, which partially agree with the presence of multiple morphotypes recovered by statistical analyses of morphological characters (Figures 3.3 to 3.6). For instance, five out of the six morphotypes at Mt Gilboa also have unique haplotypes and all four morphotypes at Tarn Cave are also genetically distinct. Similarly, each of the two morphotypes identified at Red Desert and Sentinel Peak represent different haplotypes. However, in some cases distinct morphotypes shared the same haplotypes, such as the TFLP and TSLG morphotypes at Coleford and Mt Gilboa, or OFL and OFLD at Verloren Valei. Based on the genetic results we can firmly reject the possibility that the variation observed at sympatric sites in *S. longicauda* is due to plasticity or the presence of multiple cohorts within a panmictic population.

Variation at the regional scale

The analyses of morphotypes at sympatric sites provides clear evidence consistent with the presence of nine different taxa that can be diagnosed through morphometrics and genetics, which is a clear departure from Hall (1982), who only recognised two varieties of *S. longicauda*. However, the presence of distinct morphotypes in sympatry does not necessarily mean that they are stable entities in time and space. We therefore also performed morphological and genetic analyses at a regional scale, considering them across their geographical range in South Africa. The results provide mixed evidence for their status as distinct evolutionary lineages. The multivariate classification analysis showed high values of *a posteriori* correct classification (Table 3.3). However, the percentage of correctly classified morphotypes was generally lower compared to the analogous analysis that was done for sympatric sites, indicating the presence of more overlap between certain morphotypes. In the particular case of TFLD and TFLP, morphological and genetic distances were close to zero, providing a likely explanation for why almost 40% of the former was misclassified as the latter in the regional scale analysis (Table 3.3). The results from the multivariate analysis revealed that, in contrast to what may be expected for a florally diverse genus, leaf length and width contributed strongly to the clustering of morphotypes (Figure 3.7A, B; Table 3.2). The importance of vegetative characters was further supported by the results from the HCA, which divided the *S. longicauda* complex into a uni- and bifoliate cluster respectively. Both the genetic distinctiveness of morphotypes that vary in leaf traits, as well as evidence from studies on model plants such as *Arabidopsis thaliana* and *Zea mays* that have shown that leaf characters such as form, margin pattern, area, curvature, and number, are determined by key genes (Hofer *et al.* 2001; Tsukaya 2002, 2005; Kessler and Sinha 2004; Nicotra *et al.* 2011; Li *et al.* 2016), suggest that leaf variation does not merely represent age differences of individual plants.

Despite the relatively clear patterns emerging from the multivariate analyses of morphological traits, only for some morphotypes congruent patterns among the HCA, haplotype network, and phylogenetic analysis, were observed. The subdivision by leaf number in the HCA is not mirrored in the phylogenetic analysis, as the two main clades are not each characterised by leaf number. Instead, accessions with one or two leaves are found across both clades. Nevertheless, some patterns emerge from the phylogenetic analysis. For instance, the accessions of OELB, OELM, and *S. rhodanthum* constitute a well-supported clade (Figure 3.9). Despite dramatic diversity in floral characters (van der Niet *et al.* 2015; Castañeda-Zárate *et al.* 2021; Chapter 2), which may explain why these morphotypes do not represent a cluster in the HCA, this clade is characterised by a leaf

character synapomorphy: the single erect leaf. Furthermore, all populations of TELW comprised a well-supported monophyletic clade. For other morphotypes, resolution is inadequate to establish monophyly, although the results do not reject it; this applies to OFL, TFLD, and TFLP. Morphotypes that are clearly not monophyletic include OFLD and TSLG. Accessions of the former are distributed across three subclades in both Clade A and Clade B. The accessions from Clade B represent miniature flowers that appear morphologically distinct and different from the other accessions in Clade A, but additional data are required to identify diagnostic traits to further subdivide the OFLD morphotypes. Accessions from the TSLG morphotype are also distributed across the two main clades. Most accessions belong to Clade B, but accessions from the morphologically and geographically distinct TSLG morphotype from Woodbush is a member of Clade A (Figure 3.9). Plants from this population differ notoriously from the remaining populations of this morphotype sampled mostly in KwaZulu-Natal. The divergent position of the Woodbush accessions may be further explained for the presence of distinctive light pink flowers that also differ in floral scent from the widespread TSLG morphotype (T. van der Niet, pers. comm.). It is likely that the Woodbush population has undergone an independent evolutionary history from the southernmost populations of TSLG, which resulted in the evolution of unique floral traits, including colour and scent, as well as leaf traits (Fenster *et al.* 2004; Raguso 2008; Schiestl and Johnson 2013; Schiestl 2015). Other accessions of TSLG that occur in both Clade A and B are due to cloning of the two ITS amplicons that produced heterogeneous sequences, indicating a possible case of hybridization, and suggesting that gene flow between progenitors from the different clades may have recently occurred (Devey *et al.* 2008; van der Niet and Linder 2008). The accession of *S. buchananii* was recovered as part of clade A. The inclusion of this species in a broad definition of the complex was suggested by Hall (1982), who argued that its morphology represents a large form of *S. longicauda*. Floral morphology resembles that of OFL and TELW, but the spurs are longer. Because of the nested positions of *S. buchananii* and *S. rhodanthum* within *S. longicauda*, the species as currently circumscribed is paraphyletic. Despite some inconsistency in patterns retrieved from the morphological and genetic analyses respectively, the Mantel test of morphological and genetic distances from sympatric and allopatric populations found a significant correlation ($r = 0.25$; $p < 0.001$; Figure 3.10), suggesting that morphological and genetic divergence occurs jointly and therefore that morphological differentiation may be a useful representation of the extent of evolutionary divergence.

Despite overall congruence between patterns of morphological and genetic divergence, the analysis presented here suggests non-monophyly of several morphotypes and the presence of clones from the same individual that group with two divergent clades. It is therefore clear that the phylogenetic

analysis based on a single gene cannot be used to draw final conclusions about the relationships among morphotypes within the species complex, especially in the light of previous incongruence between ITS and plastid sequences within the *S. longicauda* species complex (van der Niet and Linder 2008; Castañeda-Zárate *et al.* 2021; Chapter 2). The challenges outlined above are a common feature of recently diverged taxa. Therefore, the exclusive use of DNA sequence data that are mostly used for resolving relationships above the species level may not represent an optimal approach for species delimitation, because of gene flow and incomplete lineage sorting. Techniques implementing Next Generation Sequencing (NGS) or High-throughput-sequencing (HTS) would help to solve the phylogenetic relationships and achieve a stable species delimitation in the *S. longicauda* complex as previously demonstrated in orchids and other plant groups (Bräutigam and Gowik 2010; Yang and Li, De-ZhuLi 2014; Wanke *et al.* 2017; Bogarín *et al.* 2018; Pérez-Escobar *et al.* 2018; Taheri *et al.* 2018; Villaverde *et al.* 2018; Frajman *et al.* 2019; Granados Mendoza *et al.* 2020). Nevertheless, the monophyly of some morphotypes, such as TELW, OELB and *S. rhodanthum* supports their status as independent evolutionary lineages.

Taxonomic implications

Floral characters have extensively been used in orchid taxonomy to delimitate and segregate groups. However, species delimitation based only on these characters sometimes produces classifications that do not reflect phylogenetic relationships (Borba *et al.* 2002; van der Niet *et al.* 2011; Pessoa *et al.* 2020). In the *S. longicauda* species complex, two varieties that differ in the length of their spurs are currently recognised (Hall 1982). Our results showed that although morphotypes overlap in certain traits they can be accurately identified using a series of both floral and vegetative characters.

Using the original descriptions of both varieties of *Satyrium longicauda* and comparing each morphotype to high-resolution digital images of the type specimens of both existing varieties, we infer that *S. longicauda* var. *longicauda* was described based on a specimen of the TSLG (Figure 3.2G). Overall morphology, flowering period and the type locality of *S. longicauda* var. *jacottetianum* indicate it was described based on an individual of the TFLD morphotype (Figure 3.2F). Contrary to the type specimen of this variety, which has three basal leaves, we have only found individuals with two leaves. Because the spurs of the type specimen were partially or fully covered by bracts or other flowers, we could not reliably obtain their lengths. Nevertheless, the original description reports a spur length of 16 mm. Using spur length, other morphotypes that could be assigned to var. *longicauda* include OELM, OFL, and TELW (Figure 3.2J). Since most individuals within OELB, OFLD,

and TFLP have shorter spurs than var. *longicauda,* they fit within the var. *jacottetianum* concept (Figure 3.2J).

Hall's (1982) assessment emphasised the variation in floral spur length as a basis for describing varieties in *S. longicauda*, and this character has been used as an important taxonomic character in *Satyrium* more broadly (Hall 1982; Johnson and Linder 1995; Johnson and Kurzweil 1998; Linder *et al.* 2005; van der Niet 2020). However, use of this single character to delimit taxa sometimes offers poor resolution by oversimplifying taxon boundaries (Morgan and Ackerman 2014). As pointed out by Hall (1982), spurs in *S. longicauda* show a broad variation in length. Indeed, we recorded spur length values ranging from 11.1 mm to 52.4 mm. If we had based our delimitation only on spur length as currently proposed, the assignment of different individuals to any of the two varieties as recognised by Hall (1982) would have resulted in single morphotypes being assigned to different varieties. For example, except for TELW and TSLG that bear the longest spurs, all other morphotypes including *S. rhodanthum*, had some individuals with spur length that could be grouped either in *S. longicauda* var. *jacottetianum* or *S. longicauda* var. *longicauda*. Besides this, the phylogenetic results further suggest that spur length is a labile trait. In addition, considering that some of the largest (TSLG) and smallest (TFLD) morphotypes group together in one clade, their phylogenetic relationships do not support the existence of two varieties as described by Hall (1982).

The delimitation of taxa recognised as part of a species complex is challenging, especially if several taxa share a similar morphology due to convergence or occur in sympatry (Borba *et al.* 2002; van der Niet *et al.* 2011; Trávníček *et al.* 2012; Huang *et al.* 2020). This is because local variants may either represent a panmictic population, or (partially) reproductively isolated entities (see below). Although orchid taxonomy has historically been based on reproductive characters for species delimitation, some studies suggest that vegetative characters outperform floral morphology for classification (Chase *et al.* 2009; Bateman and Rudall 2011; Salazar and Dressler 2011). Hall (1982) suggested the potential recognition of different sympatric groups with identical floral morphology but variable number and position of leaves at the forma infraspecific rank. He specifically considered the possibility that sympatric variants with different leaves may be characterised by single gene polymorphism. Our results reject this possibility, given the extensive variation observed in ITS sequences among sympatric variants. The evidence presented here indicates that apart from *S. rhodanthum* that is already recognised as a valid species different from the rest of the complex, the OELB and TELW morphotypes could already be unambiguously assigned independent taxonomic

status (Hamilton and Reichard 1992), whereas for several other morphotypes, such as OFL, this is likely valid but awaits further phylogenetic support.

Our subdivision that considers morphotypes of *S. longicauda* based on diagnostic leaf characters, habitat, pollination systems, and spur length represents a classification framework that has identified more diagnosable morphotypes than the varietal classification proposed by Hall (1982). We consider that more studies are required before proposing a stable classification of the complex. The identification of the morphotypes of *S. longicauda* recognised here can be achieved by looking at the number, position, and size of leaves of individuals that are not flowering, or from herbarium specimens (provided the leaves were collected), highlighting how the use of non-floral characters may be advantageous. Indeed, as indicated by the names given to each morphotype (Figure 3.2), we classified each population based on the qualitative discrimination of vegetative characters, overall size, and habitat, rather than quantitative characters (morphometrics). Although pollination system was used to differentiate morphotypes with one erect leaf, both OELB and OELM morphotypes also contain unambiguous unique morphological characters including variation in flower size and shape which allow their accurate delimitation (see Chapter 3).

Field studies along with implementation of methods for studying populations rather than isolated individuals offer several advantages over herbarium-based taxonomy for the correct diagnosis and identification of taxa that often remain hidden as part of species complexes. Based on the results of both morphometrics analyses and ITS sequences, plus the observation of recurrent coexistence of morphotypes that are potentially reproductively isolated, the re-circumscription of *S. longicauda* is clearly necessary. Our study provides a starting point for the recognition of several potential incipient species (potentially at least quadrupling the number of known taxa within the complex). However, splitting the *S. longicauda* species complex seems premature at this stage. A broader sampling that covers the vast geographical distribution where the complex has been reported, coupled with analyses of multiple sources of evidence (e.g., morphology, ecology, genetics, flowering phenology) is required. This approach will undoubtedly contribute to gain a better understanding of the taxonomy of the complex. Last, but not least, it will unravel the presence of unexpected endemic or unique taxa (Mulcahy 2008; Trávníček *et al.* 2012) contributing to re-assessment of taxon boundaries. This is important not only for fields such as taxonomy and evolutionary biology, but also of crucial significance for regional biodiversity conservation (Barrett and Freudenstein 2011; Bateman and Rudall 2011).

Coexistence of multiple closely related morphotypes

The presence of multiple morphotypes, which appear to represent distinct evolutionary lineages, and which often co-occur in sympatry, begs the question as to what mechanisms underpin reproductive isolation between these entities. On the one hand, prezygotic barriers that limit interspecific gene flow between congeners could explain why multiple morphotypes can coexist. Such prezygotic barriers could include occurrence in specific habitats (Schnitzler *et al.* 2011; Anacker and Strauss 2014; Mantel and Sweigart 2019), divergent flowering phenology (Anderson *et al.* 2009; Newman *et al.* 2012; Matsumoto *et al.* 2019; Ramírez-Aguirre *et al.* 2019; Osborne *et al.* 2020), use of different pollinators or divergent use of the same pollinator (Peakall *et al.* 2010; Pedron *et al.* 2012; Ramírez-Aguirre *et al.* 2019), and divergence in mechanical features leading to character displacement (Queiroz *et al.* 2015; Zheng *et al.* 2017; Newman and Anderson 2020). Studies on the reproductive biology of *S. longicauda* have shown that TELW, TSLG, and TFLP are visited by nocturnal moths that differ in the length of their proboscis (Harder and Johnson 2005; Jersáková and Johnson 2007; Ellis and Johnson 2010; Johnson *et al.* 2011, 2019; Duffy and Johnson 2014). More recently, Castañeda-Zárate *et al.* (2021) reported the pollination systems of the six sympatric morphotypes occurring at the Mt Gilboa site which exhibit partial overlapping flowering (Chapter 2). Apart from the OELB morphotype that is pollinated by oil-collecting bees, the remaining five morphotypes including OELM, OFL, TELW, TFLP, and TSLG are mainly pollinated by moths, although OELM and TELW are also occasionally visited by long-tongued flies. In addition, pollination by sunbirds in *S. rhodanthum* has been demonstrated (van der Niet *et al.* 2015). Pollinator observations therefore only partly explain pollinator-mediated reproductive isolation, suggesting operation of additional isolating barriers, given the absence of obvious hybrids in the field.

Postzygotic barriers including polyploidization and genome rearrangement also have the potential to lead to reproductive isolation via genetic incompatibilities. These mechanisms operate in case stigmas are reached by interspecific pollen by preventing hybridization, or through the formation of nonviable progeny (Ramsey and Schemske 1998; Soltis *et al.* 2003; Hersch-Green 2012; Kolář *et al.* 2017). Indeed, polyploidy speciation is frequent in plants (Alix *et al.* 2017) , and natural hybridization between *Satyrium* species has been documented (Johnson 2018). A preliminary study of ploidy levels on *S. longicauda* revealed that most populations are represented by diploid individuals, whereas polyploid populations were present but uncommon (Dreteler K, unpublished data; van der Niet, T, pers. comm.). Although prezygotic barriers that act synergistically seem to explain the co-existence of different morphotypes of *S. longicauda* through different flowering time and the use of

different specialised pollinators as oil-collecting bees, sunbirds in the case of *S. rhodanthum*, or moths with different mouthpart length, it is necessary to perform controlled crosses in order to quantify the contribution of postzygotic barriers. Therefore, further work quantifying isolating mechanisms among the morphotypes identified here is required to understand the role of prezygotic and postzygotic isolation barriers in maintaining the identity of discrete taxa that not only co-exist but sometimes also co-flower. The resultant evidence from the suggested experiments and the morphological and genetic evidence presented here will allow a more complete reassessment of the complex which will ultimately be reflected in a stable classification.

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Supplemental Information

Table S3.1. Study populations of the morphotypes of *Satyrium longicauda* and *Satyrium rhodanthum*. Acronyms for morphotypes: OELB= One Erect Leaf-Bee; OELM= One Erect Leaf-Moth; OFL= One Flat Leaf; OFLD= One Flat Leaf-Dwarf; TELW= Two Erect Leaves-Wetland; TFLD= Two Flat Leaves-Dwarf; TFLP= Two Flat Leaves-Pink; TSLG= Two Spreading Leaves-Giant. Acronyms for provinces: EC= Eastern Cape; FS = Free State; NL= KwaZulu-Natal; LP= Limpopo; MP= Mpumalanga. Vouchers of all morphotypes included in this study were collected by the first author (MCZ) unless otherwise stated and are housed in the Bews Herbarium (NU) at the University of KwaZulu-Natal. Collector's number and date of sampling in bold are linked to the specimen used for DNA extraction. Number of individuals used for morphometrics analyses (M) and DNA sequences (G) are separated by a forward slash (/). Sample size with a superscripted letter ([c]) refers to cloned sequences. GenBak sequences obtained by or used in van der Niet and Linder (2008) are used here as outgroup including two clonal sequences. Herbarium acronyms are given according to Thiers (2020). Geographical coordinates of *S. rhodanthum* populations (including co-existing morphotype) are omitted as the species is enlisted as Endangered (EN) in the Red List of South African Plants (Mtshali 2015).

Site (Province)	Coordinates	Morphotype	Voucher	Date of sampling	Sample size (M/G)	ITS GenBank Accession
Satyrium longicauda Lindl.						
Bushman's Nek (NL)	29°51'29.06"S 29°10'29.24"E	TSLG	1420, 1522, 1563	8 January 2017, 14 January 2018, 19 January 2018	29/0	—
		TFLP	**T807**, 1564, 1565	**2 February 2014**, 19 February 2018	38/3	MT586415-MT586417
Byrne (NL)	29°48'16.08"S 30°11'28.99"E	OFL	1668	7 December 2018	1/0	—
Castleburn (NL)	29°44'27.09"S 29°18'27.61"E	TFLP	1584	29 March 2018	27/0	—
Coleford Nature Reserve (NL)	29°57'28.58"S 29°27'42.51"E	TSLG	**1656**	**8 February 2019**	24/1+2[c]	MT586393, MT586435, MT586436
		TFLP	**1651**	**8 February 2019**	33/2	MT586418,

						MT586419
Garden Castle Nature Reserve (NL)	29°44'51.00"S 29°12'31.64"E	OELM	**T560**, 1683	**16 December 2012**, 19 January 2020	42/3	MT586323-MT586325
Giant's Castle Nature Reserve (NL)	29°17'51.96"S 29°29'54.07"E	OFLD	1618, 1623	27 December 2018, 3 January 2019	52/0	—
Graskop (MP)	24°56'8.89"S 30°50'46.93"E	TELW	1666	27 March 2019	3/0	—
Gxwalingwenya (NL)	29°38'33.60"S 29°22'56.12"E	TFLP	1712	25 February 2020	3/0	—
Highflats (NL)	—	TSLG	**1497**	**31 December 2017**	43/3	MT586394-MT586396
Highmoor Nature Reserve (NL)	29°19'9.37"S 29°36'47.92"E	OELM	1684, 1686	20 January 2020	2/0	—
		OFL	**1638**	**31 January 2019**	8/3	MT586341-MT586343
		OFLD	1639	31 January 2019	1/0	—
		TSLG	1642	6 February 2019	31/0	—
		TFLP	**1571**	**6 March 2018**	12/3	MT586420-MT586422
Hlatikulu Crane and Wetland Sanctuary (NL)	29°16'30.61"S 29°39'55.22"E	TFLP	1576, 1585	24 March 2018, 30 March 2018	20/0	—
Hogsback (EC)	32°36'5.60"S 26°55'2.20"E	TSLG	**1566**	**20 February 2018**	5/3	MT586397-MT586399
Inzinga (NL)	29°31'35.61"S 29°38'56.68"E	TFLP	1580	26 March 2018	8/0	—
Jolivet (NL)	—	TSLG	**1609**, 1619	**13 December 2018**, 29 December 2018	38/2	MT586400, MT586401
Kamberg Nature Reserve (NL)	29°22'37.46"S 29°43'10.73"E	OELM	**T555**, 1503	**15 December 2012**, 1 January 2018	37/4	MT586326-MT586329
		TSLG	1518, 1543,	9 January 2018, 27 January 2018,	30/0	—

			1550, 1552	5 February 2018, 10 February 2018		
		TFLP	1445, 1449, 1451, 1455, 1551, 1569	25 February 2017, 8 March 2017, 9 March 2017, 13 April 2017, 10 February 2018, 24 February 2018	49/0	—
Lotheni Rd (NL)	29°31'21.51"S 29°42'36.11"E	TFLP	1645	7 February 2019	17/0	—
Maluti-a-Phofung (FS)	28°31'19.07"S 29°11'28.59"E	TFLP	1711	20 February 2020	3/0	—
Mooi River (NL)	29°12'55.27"S 29°44'42.20"E	TSLG	**T609**	**27 January 2013**	1/3	MT586407-MT586409
Mount-Aux-Sources (NL)	28°45'5.51"S 28°53'15.52"E	OFLD	**T608,** 1434	**25 January 2013,** 24 January 2017	18/3	MT586358-MT586360
Mpofana Rd (NL)	29°25'51.30"S 29°45'23.29"E	OFL	1422	9 January 2017	8/0	—
		TSLG	1423, 1424	9 January 2017	5/0	—
Mt Gilboa Nature Reserve (NL)	29°17'10.49"S 30°17'33.32"E	OELB	**T550**, 1412, 1414, 1488, 1494, **1606**, 1614	**13 December 2012**, 6 December 2016, 18 December 2016, 13 December 2017, 17 December 2017, **6 December 2018**, 19 December 2018	60/4	MT586319-MT586322
		OELM	**T605**, 1490, **1495**, 1612, 1622	**6 January 2013**, 13 December 2017, 20 December 2017, 17 December 2018, 31 December 2018	87/5	MT586330-MT586334
		OFL	**T606**,	**6 January 2013**,	95/5	MT586344-

			1413, **1506**, 1508, 1613	18 December 2016, **2 January 2018**, 2 January 2018, 17 December 2018		MT586348
		TELW	1415, 1426, **1523**, 1620, 1635	18 December 2016, 17 January 2017, **17 January 2018**, 30 December 2018, 23 January 2019	53/3	MT586375-MT586377
		TSLG	**T607**, **1425**, 1444, 1537, 1547, 1634	**6 January 2013**, **17 January 2017**, 7 February 2017, 26 January 2018, 1 February 2018, 23 January 2019	78/5	MT586402-MT586406
		TFLP	1443, 1452, **1453**, 1641	4 February 2017, 10 March 2017, **5 April 2017**, 2 February 2019	50/6	MT586423-MT586428
Ngome Forest Reserve (NL)	27°50'18.75"S 31°21'7.35"E	OELM	1476, 1501	5 November 2017, 16 November 2017	8/0	—
Annex Cave (NL)	29°43'36.72"S 29°10'26.36"E	OFLD	**T713**	**28 December 2013**	12/3	MT586355-MT586357
Queen Elizabeth Park (NL)	29°34'13.75"S 30°19'17.55"E	TSLG	1621, 1691	30 December 2018, 22 January 2020	32/0	—
Red Desert Nature Reserve (NL)	31°3'52.89"S 30°11'46.80"E	TELW	**1598**, 1603	**9 September 2018**, 21 October 2018	22/3	MT586378-MT586380
		TSLG	**1602**	**21 October 2018**	20/2	MT586410, MT586411
Sani Pass (NL)	29°35'12.73"S 29°17'31.08"E	OELM	1628	4 January 2019	15/0	—
		OFLD	1421	9 January 2017	16/0	—

Sentinel Peak (NL)	28°43'10.82"S 28°53'48.00"E	OFLD	**T806**, 1435, 1616	**2 February 2014**, 26 January 2017, 23 December 2018	32/3	MT586361-MT586363
		TFLD	**T805**, 1436	**29 January 2014**, 28 January 2017	27/3	MT586387-MT586389
Tarn Cave (NL)	24°51'32.08"S 29°8'14.51"E	OELM	1419, 1510, 1608, **T1048** **1630**	8 January 2017, 5 January 2018, 12 December 2018, **12 January 2019**, **12 January 2019**	55/3	MT586335-MT586337
		OFL	1418, 1511, **1526**, **T1046** 1631	8 January 2017, 5 January 2018, **19 January 2018**, **12 January 2019**, 12 January 2019	48/3	MT586349-MT586351
		OFLD	**1416**, 1512, 1525, **T1047**, 1632	**8 January 2017**, 5 January 2018, 19 January 2018, **12 January 2019**, 12 January 2019	47/3	MT586364-MT586366
		TFLD	1417, 1441, 1527, **T1048**, **1629**	8 January 2017, 31 January 2017, 19 January 2018, **12 January 2019**, **12 January 2019**	43/3	MT586390-MT586392
uMkhomazi Nature Reserve (NL)	29°29'23.97"S 29°42'54.29"E	TFLP	1577	26 March 2018	14/0	—
Umtamvuna Nature Reserve (NL)	30°59'36.83"S 30°11'3.44"E	OELM	1469, **1472**, 1601	29 October 2017, **4 November 2017**, 21 October 2018	30/3	MT586338-MT586340
		TELW	1463, 1467,	28 September 2017, 17 October 2017,	60/0	—

			1502, 1600	28 October 2017, 21 October 2018		
		TSLG	1471, 1475, 1477, 1480	4 November 2017, 5 November 2017, 20 November 2017, 26 November 2017	30/0	—
Verloren Valei Nature Reserve (MP)	29°17'3.56"S 30°8'54.55"E	OFL	**T751**, 1429, 1690	**26 January 2014**, 21 January 2017, 21 January 2020	33/3	MT586352-MT586354
		OFLD	**1430**	**21 January 2017**	6/2	MT586367, MT586368
		TELW	**T750**, 1427	**21 January 2017**	20/3	MT586381-MT586383
		TSLG	**BB2253**, 1428	**26 January 2002**, 21 January 2017	26/2[c]	**EF601501**, **EF601502**
Vernon Crookes Nature Reserve (NL)	30°16'12.29"S 30°37'11.23"E	TSLG	1607, 1615	10 December 2018, 21 December 2018	38/0	—
Wahroonga (NL)	29°35'51.37"S 30°9'6.70"E	TSLG	**T610**, 1532	**28 January 2013**, 19 January 2018	52/3	MT586412-MT586414
Witsieshoek (FS)	28°41'7.74"S 28°54'8.31"E	OFLD	1440, **1546**	29 January 2017, **29 January 2018**	30/6	MT586369-MT586374
Woodbush Forest Reserve (LP)	23°48'16.69"S 29°58'17.49"E	TSLG	**1545**	**29 January 2018**	3/3	MT586384-MT586386
Satyrium rhodanthum Schltr.						
Highflats (NL)	—	—	**T712**, 1483, 1498	**25 December 2013**, 10 December 2017, 31 December 2017	32/3	MT586429-MT586431
Hlutankungu (NL)	—	—	**T711**, 1485, 1492, 1611	**15 December 2013**, 16 December 2017, 10 December 2018, 13 December 2018	20/3	MT586432-MT586434
Jolivet (NL)	—	—	1470,	1 November 2017,	20/0	—

	1605 16 November 2018	
	Outgroup	
Species	Locality (voucher specimen; herbarium)	ITS GenBank Accession
Satyrium bicorne (L.) Thunb.	Romans river, South Africa (T46; BOL, Z)	AY704978
Satyrium buchananii Schltr.	Nyika Plateau, Malawi (HK2053; MAL)	AY704982
Satyrium monadenum Schltr.	Nyika Plateau, Malawi (T173; MAL, Z)	EF601507
Satyrium neglectum Schltr.	1. Kitulo Plateau, Tanzania (T417; DSM, Z)	EF601512
	2. Mount Mulanje, Malawi (T200; MAL, Z)	EF601511
	3. Mount-Aux-Sources, South Africa (T164; Z)	EF601509
	4. Naude's Nek Pass, South Africa (T206; PRE, Z)	EF601510
Satyrium sceptrum Schltr.	1. Nyika Plateau, Malawi (HK1985; MAL, UZL)	AY704999
	2. Londiani, Kenya (T272; Z)	EF601527
	3. Mbeya Peak, Tanzania (T403; DSM, Z)	EF601528

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CHAPTER 4

ANALYSIS OF ASSOCIATIONS BETWEEN POLLINATION NICHES AND FLORAL TRAITS IN THE *Satyrium longicauda* (ORCHIDACEAE) SPECIES COMPLEX

Abstract

Patterns of variation in floral trait syndromes associated with pollination by different major functional pollinator groups such as birds, bees, moths, or vertebrates are well-known. However, it is less clear whether intraspecific patterns of trait variation can be linked with quantitative variation in pollinator assemblages. Here I study the pollination systems of morphotypes within the *S. longicauda* complex using a combination of plant-pollinator network analysis to determine pollination niches and quantitative uni- and multi-variate analyses of floral traits. Although most morphotypes were specialised for nocturnal moth pollination, diurnal pollination either by long-tongued flies, oil-collecting bees or sunbirds was also recorded. Pollination network structure revealed five modules representing pollination niches involving oil-collecting bees, sunbirds, hawkmoths, settling moths, and a combination of long-tongued flies, moths, and sunbirds. Spur length correlated significantly with mean pollinator proboscis length. Floral scent headspace analyses revealed high volatile emission rates for all modules, apart from the sunbird pollination module. Similarly, white or pink flower colour was associated with the modules characterised by predominant insect pollination, whereas red flowers characterised the sunbird pollination module. Finally, nectar volume and concentration differed significantly among modules. Nectar volume per flower was largest for the sunbird pollination module, was larger in plants pollinated by hawkmoths than in those pollinated by settling moths, and almost zero in plants pollinated by oil-collecting bees. Although there is clear variation in floral traits among modules characterised by distinct pollinators groups, the separation of the two moth-pollination niches is mainly associated with morphological variation in spur length and nectar features, whereas colour and scent overlap between both these pollination niches.

Key words: pollination network, modularity, moth pollination, nectar, plant-pollinator interaction, colour, pollination niche, scent, spur length.

Introduction

Pollination by animals is considered one of the most important factors involved in the diversification of flowering plants (Stebbins 1970; Hernández-Hernández and Wiens 2020). Two complementary lines of evidence provide support for this idea – trait-pollinator correlations and evidence for frequent shifts between pollinators (Grant and Grant 1965; Wolfe and Sowell 2006; Cronk and Ojeda 2008; Martén-Rodríguez *et al.* 2009; Reynolds *et al.* 2009; van der Niet *et al.* 2010; Vogel 2012; Shrestha *et al.* 2013; Ashworth *et al.* 2015; Katzer *et al.* 2019). Floral traits often show co-variation among species in a manner that is associated with differences in the functional group that pollinates a species (Fenster *et al.* 2004), especially for plant species that are characterised by specialised pollination systems (Johnson and Steiner 2000). Predictable co-variation occurs specifically in floral traits such as colour, scent, nectar, and morphology. These traits are thought to be under pollinator-mediated selection, based on pollinator perception and morphology (Kulbaba and Worley 2012, 2013; Schiestl and Johnson 2013). Given the diversity of pollinator morphologies and sensory perception, specific suites of floral traits, often referred to as pollination syndromes, likely represent adaptations to different functional pollinator groups.

Both macro- and microevolutionary studies have confirmed pollinator-driven divergence by identifying frequent pollinator shifts (Johnson *et al.* 1998; Kay *et al.* 2005; Whittall and Hodges 2007; Smith *et al.* 2008; Smith 2010; van der Niet and Johnson 2012; Lagomarsino *et al.* 2017). Macroevolutionary studies have identified a pattern of frequent pollinator shifts through phylogenetic studies using a combination of pollinator observations and floral trait data (van der Niet 2021). Microevolutionary studies have zoomed in on the process of pollinator-driven divergence by focusing on cases whereby populations of the same species are characterised by an association of floral trait differences with pollination by different functional pollinator groups, resulting in the identification of pollination ecotypes (Robertson and Wyatt 1990; Johnson 1997a; Johnson and Steiner 1997; Anderson *et al.* 2010; Boberg *et al.* 2014; Sun *et al.* 2014; van der Niet *et al.* 2014; Cosacov *et al.* 2014; Newman *et al.* 2014, 2015; Peter and Johnson 2014; Parker *et al.* 2017; Trunschke *et al.* 2019; Castañeda-Zárate *et al.* 2021; Chapter 2).

Most studies investigating pollinator-driven divergence have focused on plant groups characterised by specialised pollination by diverse groups of insects, such as bees, moths, flies,

beetles, and butterflies, or various vertebrate pollinators including birds, bats, non-flying mammals and reptiles (Faegri and van der Pijl 1979; Grant 1993, 1994; Vogel 2012). Floral traits of species pollinated by these disparate pollinator taxa often covary in discrete ways (but see Goldblatt *et al.* 2004; Castañeda-Zárate *et al.* 2021; Chapter 2). For instance, bird-pollinated flowers are often red, wide, unscented and produce copious amounts of dilute nectar, whereas moth-pollinated flowers are often white, narrow, scented and produce smaller amounts of concentrated nectar (Faegri and van der Pijl 1979; Baker and Baker 1983; Grant 1993; Manning and Snijman 2002; Streisfeld and Kohn 2005; Dobson 2006a; Streisfeld and Kohn 2007; Willmer 2011; Sheehan *et al.* 2012; Vogel 2012; Byers *et al.* 2014; van der Niet *et al.* 2015; Rodrigues *et al.* 2018).

The theory underpinning the evolution of pollinator-driven divergence predicts that subtle quantitative variation in pollinator assemblages could equally lead to the evolution of floral traits, provided that pollinators differ in their sensory perception and/or morphology. Although this has been less frequently studied, some studies have revealed trait variation among species or populations characterised by minor variation in pollination systems. A characteristic pattern involves variation in the length of the nectar-bearing organ in association with variation in pollinator mouthpart length (Steiner and Whitehead 1990, 1991; Goldblatt and Manning 2000; Alexandersson and Johnson 2002; Anderson and Johnson 2007, 2009; Pauw *et al.* 2009; Anderson *et al.* 2014; Boberg *et al.* 2014; Kahnt *et al.* 2019; van der Niet 2021). For example, variation in the spur length of the two ecotypes of the orchid *Platanthera ciliaris* is associated with differences in proboscis length of their particular swallowtail butterfly pollinator species (Robertson and Wyatt 1990). Other floral syndrome traits may also vary among species with slightly different pollination systems. Corolla length, nectar concentration, and scent composition all differed between ecotypes of *Gladiolus longicollis* pollinated by short- and long-tongued hawkmoths respectively (Anderson *et al.* 2010). Similarly, two ecotypes of *Bulbophyllum ecornutum* subsp. *verrucatum* characterised by their distinct lip morphology and size, floral colour, and main floral attractants receive pollination by different species of fruit flies (Tan and Nishida 2005; Tan *et al.* 2021). Even plants displaying relative generalist pollination systems such as members within Brassicaceae show differences in corolla shape, and colour which are strongly related to different pollination niches (Gómez *et al.* 2015, 2016). However, associations between floral traits and pollinators are not always clear. For example, in *Platanthera bifolia* scent bouquets of short- and long-spurred

ecotypes showed no clear differences in spite of being pollinated by different groups of moths (Tollsten and Bergstrom 1993).

The concept of the pollination niche is useful in several ways to study pollinator-driven divergence in a quantitative framework (Phillips *et al.* 2020). Characterisation of pollination niches starts with direct observations of floral visitors, to identify whether species act either as legitimate pollinators or illegitimate floral visitors that do not participate of the pollination process. Observations can then be used for quantifying pollination niches through plant-pollinator network analyses. In particular, network modularity can be used to evaluate whether a network is formed by smaller systems or modules (Newman 2004, 2006; Newman and Girvan 2004; Olesen *et al.* 2007). Each module can be considered to reflect specialisation for pollination by a particular species or group of species (Dicks *et al.* 2002; Olesen *et al.* 2007; Danieli-Silva *et al.* 2012), and hence be representative of pollination niches. In general, niche occupancy is expected to be determined by the presence of particular functional traits (Sutherland and Vickery 1993; Alexandersson and Johnson 2002; Anderson *et al.* 2010; van der Niet *et al.* 2010; Byers *et al.* 2014; Heiduk *et al.* 2016; Parker *et al.* 2017). In case of pollination niches, this leads to an expectation of an association between variation in pollination niches (i.e. network modules) and floral trait variation, with a particular focus on traits that have potential effect on the attraction of pollinators or the mechanical-fit between flowers and pollinators (Phillips *et al.* 2020).

Here, I implement the niche framework to investigate the potential existence of pollination ecotypes in the species complex *Satyrium longicauda*. This species is an excellent study system to evaluate whether, and to what degree floral traits differ among forms that vary in magnitude of differences in pollination niches: It is morphologically diverse, and encompasses forms pollinated by sunbirds, forms pollinated by oil-collecting bees, and forms pollinated by a variety of different moth species.

Satyrium Sw. is a genus of terrestrial orchids characterised by its non-resupinate flowers provided with two spurs projecting from the hooded labellum, and a gynostemium with a long column part and a pendent anther (Hall 1982; Kurzweil and Linder 1999, 2001). Rostellum structure plays an important function by placing pollinaria on specific body parts of pollinators (Johnson 1997b). The striking diversity in floral morphology, colour, scent, and reward, reflects the extensive radiation of

pollination systems that includes autonomous self-pollination (van der Niet 2018), and biotic pollination by beetles, bees, carrion flies, long-proboscid flies, butterflies, moths, and sunbirds (e.g. Johnson 1996, 1997; Johnson *et al.* 2007, 2011). Variation among functional pollinator groups in *Satyrium* is associated with differences in floral traits including morphology (particularly spur length and rostellum structure), floral shape, colour, scent, and the type and amount of reward (van der Niet *et al.* 2010; Johnson *et al.* 2011). However, there is also tremendous variation in floral shape, spur length, nectar characteristics and scent among species sharing the moth pollination system (van der Niet *et al.* 2010; Johnson *et al.* 2011; van der Niet, Jürgens, *et al.* 2015), but the association between pollination by different types of moths (e.g. hawkmoths and settling moths) and floral traits has not yet been investigated.

Satyrium longicauda Lindl. is a common species in Southern African grasslands (Hall 1982) and it is highly diverse in several floral traits, including colour, scent, and morphology, in particular the length of the spurs that vary from 11 mm to 52 mm in length (Chapter 3). Due to the variation in floral and vegetative morphology, the species represents a complex comprising eight morphologically discrete forms (morphotypes), and *S. buchananii* and *S. rhodanthum* (Hall 1982; Kurzweil and Linder 1999; Chapter 3). Morphological variation is matched by diversity in pollination systems, including pollination by long-proboscid flies, sunbirds, oil-collecting bees, and moths (e.g. Johnson *et al.* 2011; van der Niet *et al.* 2015; Castañeda-Zárate *et al.* 2021; Chapter 2). Females of the oil-collecting bee species *Rediviva neliana* pollinate flowers while inserting their forelegs to dab the oil offered in the hairs that cover the inner surface of the labellum (Castañeda-Zárate *et al.* 2021; Chapter 2). Pollination by long-proboscid flies takes place when flies insert their tongues into the spurs to reach the nectar and touch the viscidium which gets firmly attached to the dorsal region of their tongue (Castañeda-Zárate *et al.* 2021; Chapter 2). In *Satyrium rhodanthum*, sunbirds insert their bill into the galea to feed on the nectar and pollinaria are removed when the upper mandible makes contact with the large viscidium (van der Niet *et al.* 2015). Several studies have reported moth pollination, which occurs when nectar-seeking moths inserts their proboscis into the spurs and the viscidium gets attached to the dorsal surface of the proboscis (Johnson *et al.* 2005, 2009; Jersáková and Johnson 2007; Ellis and Johnson 2010; Duffy and Johnson 2014; Castañeda-Zárate *et al.* 2021; Chapter 2). The available pollination studies thus suggest that there is considerable variation in pollinator assemblages. Therefore, *S. longicauda* represents an ideal system to evaluate whether floral traits not only vary among pollination niches

characterised by discrete functional pollinator groups, but also among pollination niches that differ in members of same functional group (i.e. nocturnal moths).

The aim of this study was to identify whether and to what extent floral trait variation in *S. longicauda* is associated with differences among and within functional pollinator groups. The objectives were 1) to characterise pollination niches; 2) to quantify floral traits, and 3) to evaluate whether variation in floral traits is associated with variation in pollination niches, both among and within functional pollinator groups.

Material and Methods

Study system and study sites

Morphotypes within the *Satyrium longicauda* (Orchidaceae) complex are easily distinguished by a combination of floral and vegetative characters (Chapter 3). As proposed by Hall (1982) and Castañeda-Zárate *et al.* (2021), *Satyrium longicauda* comprises eight morphotypes grouped either as *S. longicauda* var. *jacottetianum* or *S. longicauda* var. *longicauda*, and another two species, *S. buchananii* and *S. rhodanthum* (Chapter 2 and Chapter 3). *Satyrium rhodanthum* was previously considered a species of hybrid origin (Hall 1982), but has recently been treated as a separate species (Linder and Kurzweil 1999; Johnson and Bytebier 2015). Moreover, it was recovered as being nested within *S. longicauda* in a phylogenetic analysis (Chapter 3).

To distinguish among the morphotypes that form part of both varieties, I use the informal groups identified by Castañeda-Zárate (Chapter 2 and Chapter 3) and Castañeda-Zárate et al. (2021), which consider the number and position of the leaves, plant size, flower colour, pollination system and habitat: one erect leaf-bee (OELB), one erect leaf-moth (OELM), one flat leaf (OFL), one flat leaf-dwarf (OFLD), two erect leaves-wetland (TELW), two flat leaves-dwarf (TFLD), two flat leaves-pink (TFLP), and two spreading leaves-giant (TSLG). Here, I sampled individuals for nine groups (eight morphotypes and one species) except *S. buchananii* for which natural populations could not be accessed. *Satyrium rhodanthum* is characterised by its faintly day-scented and bright red flowers that offer nectar as reward (van der Niet *et al.* 2015). The remaining eight morphotypes are characterised by flowers that produce a sweet scent all day long; six morphotypes have purely

white flowers or white flowers that are tinged with light pink and two are characterised by pink to reddish flowers (Chapter 3). They all offer nectar as reward, except one morphotype which offers fatty acids instead (Castañeda-Zárate *et al.* 2021; Chapter 2). For convenience, hereinafter I refer to all units included in this study (including *S. rhodanthum*) as *Satyrium longicauda*, which is consistent with the fact that *S. rhodanthum* makes *S. longicauda* paraphyletic (Chapter 3).

Fieldwork was carried out during the flowering season comprising two main periods: from December 2012 to February 2014 and from January 2017 until December 2019. Coastal populations start flowering during late winter (August), and flowering ends in autumn (April) for some morphotypes of inland populations. Fifty-five populations of the eight morphotypes of *S. longicauda* distributed across thirty-two sites, and three populations of *S. rhodanthum*, two of which occur in sympatry with *S. longicauda*, were sampled. All populations occur in South Africa, but are distributed across five provinces. Forty-seven populations are found in KwaZulu-Natal, four in Mpumalanga, two in the Eastern Cape, one in the Free State, and one in Limpopo. Details of voucher specimens, field sites and sample sizes for methods described below are given in Table S4.1.

Visitor observations

To determine pollen vectors of *S. longicauda*, I implemented direct observations and set up motion-trigger cameras to record flower visitors at several sites (Table S4.1). Visitor observations were conducted in 1-5 populations per morphotype. Patches of approximately 5-10 m^2 with several dozens to hundreds of flowering individuals were selected. Depending on accessibility, observations were carried out during at least three days from 8:00 to 20:00 h in order to identify main activity hours of visitors. To increase the chances of documenting visitor species, motion trigger cameras (Bushnell NatureView HD Cam Model #: 119740, USA) were also used. These cameras have been shown to be effective for recording both hawkmoth and noctuid flower visitors (Johnson *et al.* 2019). Cameras were placed at distances of 460 or 250 mm respectively, from the target plant. However, this method could not be used at all sites due to the risk of losing cameras or accommodation restrictions. Thirty-five populations of *S. longicauda*, occurring in eighteen different sites, were visited for direct pollinator observations and/or camera trapping (Table S4.1). Additionally, to determine the time of day at which plants are visited (and potentially

pollinated), we selected fifteen or more individuals with unvisited flowers, as identified by the presence of both pollinaria, and marked their bracts with a permanent marker as an indication of pollinarium presence. Marked flowers were inspected during three consecutive days in order to register the timing of pollinarium removals. For diurnal pollination (7:00 to 17:59 h) the number of pollinaria removed was registered at 18:00 h while evidence for nocturnal pollination (18:01 to 06:59 h) was evaluated at 7:00 h.

Once the timing of visitation was known, subsequent observations were mostly focused between 8:00 to 14:00 h for diurnal visitors and between 18:00 to 20:00 h for nocturnal visitors respectively. Observations of nocturnal visitors were conducted with a head lamp and a spotlight with a red filter to avoid disturbing foraging behaviour of visitors. Any insect found visiting flowers of *S. longicauda* was caught when possible by using a hand net, killed in a jar that contained ethyl acetate fumes, and pinned for identification. Caught insects were inspected for the presence and number of pollinaria or viscidia, and the body structure to which they were attached was recorded. In addition, proboscis length, defined as the distance from the tip to the area between the labial palps was measured in all Lepidoptera and from the tip to the base of the labium in Diptera. Floral visitors were classified either as "visitors" or "pollinators". The former includes all species that attempted to feed on nectar of the flowers but were never observed to carry pollinaria, whereas the latter only includes species that acted as pollen vectors, using the presence of pollinaria as a proxy. Insect specimens were identified with the aid of relevant literature (e.g. Pinhey 1975) and consultation with specialists (see acknowledgements). Insect collections for this study are deposited at the KwaZulu-Natal Museum, in Pietermaritzburg (South Africa).

Characterisation of pollination niches

Following Phillips et al. (2020), I characterised pollination niches based on modularity as a measure of species interaction organisation (Olesen *et al.* 2007; Carstensen *et al.* 2016). To determine the number of modules representing potential pollination niches, a weighted interaction matrix with plant morphotypes in rows and pollinator (not visitor) species in columns (as recorded through direct observation and camera trapping) was created. The data was pooled for each plant morphotype in case pollination activity was determined for multiple populations. Additional pollination data of the populations studied here was extracted from the literature (Johnson *et al.*

2011; van der Niet *et al.* 2015). The number of potential pollination niches was determined through a modularity analysis. Modularity (*Q*) is a metric of network structure designed to measure the strength of grouping of a network into modules (Newman and Girvan 2004). A network in which the units form discrete interaction compartments, with little interactions between units outside of each compartment, is a modular network (Olesen *et al.* 2007). The DIRTLPAwb+ algorithm, an improvement to the LPAb+ algorithm (Liu and Murata 2010), was used for the modularity analysis, since it offers consistent results when estimating modularity in bipartite networks (Beckett 2016). The observed *Q* value was compared against a null model derived from 1,000 randomised networks (Vázquez *et al.* 2007; Cordeiro *et al.* 2020) using the *vaznull* function. Modularity was calculated in R version 4.1.0 (R Core Team 2021) with the package *bipartite* version 2.16 (Dormann *et al.* 2008, 2009). Once the number and configuration of the modules was reconstructed, floral traits including spur length, scent, colour, and nectar volume and concentration were analysed using modules as units of comparison, with the aim to identify possible associations between pollination niches and floral traits (see below).

Association between spur and proboscis length

To determine whether the floral and pollinator traits are matching, I evaluated the relationship between insect proboscis and spur length through regression analysis. *Satyrium rhodanthum* was excluded because it is pollinated by sunbirds that only insert the apical part of the bill into the galea, while the protruded tongue is used to drink the nectar present in the spurs (van der Niet *et al.* 2015); therefore, there is a lack of trait matching between spur length and bill length of the pollinator. The OELB morphotype was also excluded because spurs are non-functional in the form pollinated by oil-collecting bees (Castañeda-Zárate *et al.* 2021; Chapter 2). All available data on moth and long-tongued fly species were used to obtain mean proboscis lengths, calculated as weighted means of the specimens caught bearing pollinaria on their proboscides. In cases where pollinators were not caught, but their identity was known, I used the proboscis grand mean calculated as the average of the means of multiple populations of that particular pollinator species. For functional spur length, which is defined as the distance between the viscidium and spur tip, I obtained the mean spur length for each population for which pollinator data were available using the dataset generated by Castañeda-Zárate *et al.* (2021) and Castañeda-Zárate (Chapter 2 and Chapter 3).

Floral scent

To determine whether floral scent composition, emission rate, and the number of volatile compounds differ among modules as well as the period that the most important pollinators characterising different modules are active, I sampled floral headspace of 1-10 individuals per morphotype at each population. Most populations were sampled during day and night, but in some only night samples were taken. Day samples were collected between 9:00 and 10:00, whereas night samples were obtained between 18:00 and 19:00 hrs (representing the peak period of nocturnal moth activity) (Johnson *et al.* 2019). Whenever possible, sampling was performed *in situ*, otherwise I used cuttings placed in water. Picking inflorescences for chemical analysis has no significant effect on the production of scent volatiles within 48 hours (M. Castañeda-Zárate, pers. obs.; L. Buthelezi, unpubl. data). After sampling inflorescences for 30 min, scent traps were sealed in glass vials and stored at -20 °C until analysis.

All samples were collected using the dynamic headspace extraction method following the procedure described in Castañeda-Zárate *et al.* (2021) and Castañeda-Zárate (Chapter 2). Each inflorescence was enclosed in a polyacetate bag (Nalophan®, Kalle GmbH Germany) and air was pumped from the bag through thermodesorption cartridges filled with 1 mg of Tenax® and 1 mg of Carbotrap® at a flow rate of 50 mL min^{-1}. For each sampling episode, a control sample (empty bag) of the surrounding air was sampled in parallel, in order to detect volatiles present in the ambient air. Scent samples were analyzed by coupled gas chromatography-mass spectrometry (GCMS) using a Varian CP-3800 gas chromatograph (Varian, Palo Alto, California, USA) with a 30 × 0.25 mm internal diameter (film thickness 0.25 µm) Alltech EC-WAX column, coupled to a Bruker 300-MS quadrupole mass spectrometer in electron-impact ionization mode at 70 eV, with the detector voltage continually adjusted by the Extended Dynamic Range (EDR) function. Cartridges were placed in a Varian 1079 injector equipped with a Chromatoprobe thermal desorption device. The flow of helium carrier gas was 1 mL min^{-1}. For thermodesorption, the injector was held at 40 °C for 2 min with a 20:1 split and then increased to 200 °C at 200 °C min^{-1} in splitless mode. Meanwhile, the gas chromatograph oven was held at 40 °C for 3 min and then ramped up to 240 °C at 10 °C min^{-1} and held there for 12 min (Shuttleworth and Johnson, 2009). Identification of compounds was done using the Bruker Workstation software Version 7.0 by comparing the

obtained Kovats retention index values (KRI) with those published from the NIST Mass Spectral Program for the NIST Spectral Library Version 2.0g.

Satyrium rhodanthum scent profiles were extracted from van der Niet *et al.* (2015) and added to the dataset, resulting in a total of 303 scent samples (Table S4.1). Multivariate analyses could not be performed for the six night samples of *S. rhodanthum* because they were characterised by complete absence of scent. These samples were therefore removed from the comparative analysis of scent composition. Because of uneven sample sizes, two different datasets were built and analysed for scent composition: 1) full dataset comprising 297 samples, and 2) populations characterised by the presence of samples taken during both day and night (179 samples). Floral scent composition was compared among modules following a series of analyses based on square-root transformed values of the proportion of each compound in the total blend. First, to reduce the dimensionality of the data and display compositional similarity in floral bouquets among samples of each module, a Nonmetric Multidimensional Scaling (NMDS) analysis based on the Bray-Curtis similarity index as distance measure was implemented. Next, to test for differences in floral scent among modules between day and night samples, a two-way permutational multivariate analysis of variance (PERMANOVA) with 10,000 permutations was conducted, only with populations sampled at day and night, also based on Bray-Curtis similarities. Finally, to identify volatiles that contribute to scent composition similiarity within modules in different sampling periods, an analysis of similarity percentages (SIMPER) was implemented in PRIMER 7 (Clarke and Gorley 2015).

To quantify scent emission rates per flower and inflorescence per minute, the peaks of each sample were integrated. For quantification, 2 µl of a 1:2000 mixture of methyl benzoate and hexane was used. Total scent emission per flower was obtained after dividing the total scent emission per inflorescence by the number of flowers of each sample. The scent emission rate per flower and inflorescence respectively, were then used to compare night emission rates among modules, and to compare day and night emission rates (205 and 195 samples respectively). This was done using a generalised linear mixed-effects model (GLMM) with a gamma probability distribution and log link function. To determine if there were differences in the number of compounds between modules associated with the sampling period, a GLMM with negative binomial probability distribution and logit link function was implemented. Both analyses were run

in SPSS 27 (SPSS Inc., Chicago, Illinois, USA), with morphotype nested within module and considered as a fixed effect, whereas population was treated as a random effect. The Kenward and Roger adjustment was used for computing the degrees of freedom and standard errors (Kenward and Roger 2009). For pairwise comparisons of means, a post-hoc test was conducted using the sequential Šidák procedure (Kirk 2013). For graphical representation the means and confidence intervals were back-transformed to the original scale.

Flower colour

To assess the extent to which flower colour varies among modules, I measured the spectral reflectance of the labellum of one fully opened flower from the middle portion of the inflorescence of individuals representing different populations. Five plants per morphotype, located at least 5 m apart were sampled from each population, unless fewer plants were available (Table S4.1). Following the protocol described in Castañeda-Zárate *et al.* (2021), one flower per individual was removed, and the labellum dissected and flattened (Chapter 2). The reflectance spectrum of the outer surface was measured, resulting in measurements for 260 individuals. Reflectance spectra were obtained using an Ocean Optics USB2000 spectrophotometer (Ostfildern, Germany) and fibre optic reflection probe (UV/VIS 400 µm) coupled with an Ocean Optics UV-VIS-NIR light source (Mini-DT-2-GS Deuterium–Tungsten–Halogen) with output between 200–2000 nm. Spectrum calibration was performed using a diffuse reflectance standard (Ocean Optics WS-1) as a white standard and a black film canister as a black standard. Spectrum caption was obtained at 0.38 nm intervals using the Ocean Optics SpectraSuite SpectroScopy Software.

For a graphical representation of the spectra, the electrical noise was removed from the raw spectra and negative values were set to zero using the R package *PAVO 2* (Maia *et al.* 2019). The visible region between 300-700 nm at 1 nm intervals (resulting in 401 reflectance values) was used for representing the mean reflectance value of each module. In addition, spectra for each individual were plotted in the Endler segment colour space (Endler 1990). Due to the complex community of pollinators of *S. longicauda*, which includes bees, flies, moths and birds identified for each of the modules, I decided to analyse floral colour using marker (inflection) points instead

of particular vision systems, as marker points can be calculated without a priori knowledge of the vision system of the pollinators (Chittka and Menzel 1992; Dyer *et al.* 2012; Shrestha *et al.* 2013).

Marker points, identified as pronounced changes in reflectance, represent a valuable method to understand colour perception of plants by their pollinators (Shrestha *et al.* 2016, 2019; Dorin *et al.* 2020). Raw spectral reflectance readings were uploaded to the online Spectral-MP software, maintaining the default parameters that include amplitude (10%), wavelength range (50 nm), and 10 points of smoothing (Dorin *et al.* 2020). For each spectrum, the number of marker points calculated ranged between zero and three. Individuals with no marker points (N = 7) were excluded from statistical analyses, resulting in 253 remaining samples. In case multiple marker points were detected, only the primary point was used. Primary marker points were associated with the biggest increase in reflectance around 400 nm for white flowers and 600 nm for red flowers. The values of the main marker points were analysed by individual one-way analysis of variance (ANOVA, using the *aov* function) with subsequent post-hoc comparisons using a Tukey´s HSD (Honestly Significant Difference) test on module as categorical factor in R version 4.1.0 (R Core Team 2021).

The number of marker points can be an indication of the complexity of the shape of spectral reflectance curves. To test for differences in the proportion of individuals having spectra with single (as opposed to multiple) marker points among modules, a generalised linear model (GLM) with binomial error using the *glm* function from the R stats package (R Core Team 2021) was implemented.

Nectar

To investigate whether nectar volume and concentration differ among modules, a single fresh, fully opened and virgin (unvisited) flower (identified by the presence of both pollinaria) from the middle portion of the inflorescence was collected, and nectar volume and concentration were measured. Nectar of both spurs was extracted by squeezing their content directly into 5 µl capillary tubes (ringcaps®, Hirschmann Laborgeräte, Germany) followed by measurement of the volume. Nectar concentration (percentage of sucrose) was determined with a hand-held refractometer (Eclipse; Bellingham and Stanley Ltd, Tunbridge Wells, Kent, UK). The number of

nectar samples collected for the nine morphotypes ranged between 30 and 233. Although I tried to sample at least fifteen individuals per population, the number of flowers varied according to the availability of plants (Table S4.1), resulting in a total of 823 samples.

To detect differences in nectar volume among modules, I used generalised linear models (GLMs) with a normal distribution and an identity link function, setting module as fixed factor and nesting population inside module. Nectar volume data was treated by adding a constant value (0.1), and log-transforming the data prior to analysis to approach a normal distribution. Post-hoc analyses were performed using Šidák's procedure for pairwise comparisons (Kirk 2013). For graphing purposes, the means and standard errors were back-transformed from the log scale. The effect of module on nectar sugar concentration was evaluated using a GLM incorporating a Gaussian distribution and identity link function. Pairwise comparison of estimated marginal means was performed by using the sequential Šidák procedure.

Results

Floral visitor observations

A total of 527 hours of direct observations and 3890 hours of camera recordings resulted in characterisation of visitors for twenty six out of the thirty five populations for which pollinator observations were performed. Time spent at the nine populations for which no floral visitors were observed included 26 hours of direct observations and 96 hours of camera recording. A total of 908 floral visitors, comprising 30 species that belong to four orders (Diptera, Hymenoptera, Lepidoptera, and Passeriformes) visited *S. longicauda* and *S. rhodanthum* (Table S4.2). Of all visitor observations, 795 (87%) represent direct observations and 115 (13%) are photographs or videos obtained from motion-activated cameras (Table S4.2). Except from 11 sunbirds (1.21%), most visitors were insects (899 = 98.79%). Sixteen out of the thirty visitor species were classified as pollinators (751 = 82.53%) based on the presence of pollinaria. Although not all individuals of the identified pollinator species carried pollinaria, more than half did (437 = 58%; Table S4.2). The OELB morphotype was exclusively pollinated during daytime, whereas OFL, OFLD, TELW, TFLD, TFLP, and TSLG were exclusively pollinated at night; OELM was pollinated during both day and night.

The OELB morphotype was pollinated by the oil-collecting bee, *Rediviva neliana*. Pollinaria attach to the tibia of the forelegs of *R. neliana* females while they collect oil from the labellum (Figure 4.1A). Moth pollination occurred in the OELM, OFL, OFLD, TELW, TFLD, TFLP, and TSLG morphotypes (Figure 4.1B, 4.1C, 4.1E-4.1I). Moths visiting these morphotypes removed pollinaria with their proboscides while drinking nectar. Although mainly pollinated by moths, the OELM morphotype was also pollinated by long-proboscid flies (Figure 4.1C) and sunbirds (Figure 4.1D). Pollinaria of this morphotype become attached to the basal part of the proboscides of the flies (Figure 4.1C) and moths but to the tip of the sunbird's bill as they probe the spurs seeking for nectar. In *S. rhodanthum*, the pollinaria get attached to the apical portion of the bill (Figure 4.1J).

Modularity

The pollination network based on interactions between *S. longicauda* morphotypes and pollinators was significantly modular based on the null model (Q_{obs} = 0.56; 95% CI $Q_{null\_vaznull}$ = 0.10-0.21). Five modules were detected, formed by one or three morphotypes and one or five pollinator species (Figure 4.2). *Satyrium rhodanthum* and two *S. longicauda* morphotypes (TFLP and OELB) represented independent modules (modules C, D and E). The TFLP morphotype is mainly pollinated by the settling moth *Cucullia hutchinsoni*, but occasionally by hawkmoths, whereas the OELB morphotype is exclusively pollinated by *Rediviva neliana* females; *S. rhodanthum* is exclusively pollinated by *Chalcomitra amethystina* sunbirds. The remaining two modules A and B consisted of three morphotypes each. The first module A includes OFL, TELW, and TSLG and is characterised by pollination by the hovering hawkmoth *Basiothia schenki*, as well as other hawkmoths, while pollination mostly by three different pollinator functional groups (long-proboscid flies, settling moths, and sunbirds) characterises module B that comprises the morphotypes OELM, OFLD and TFLD.

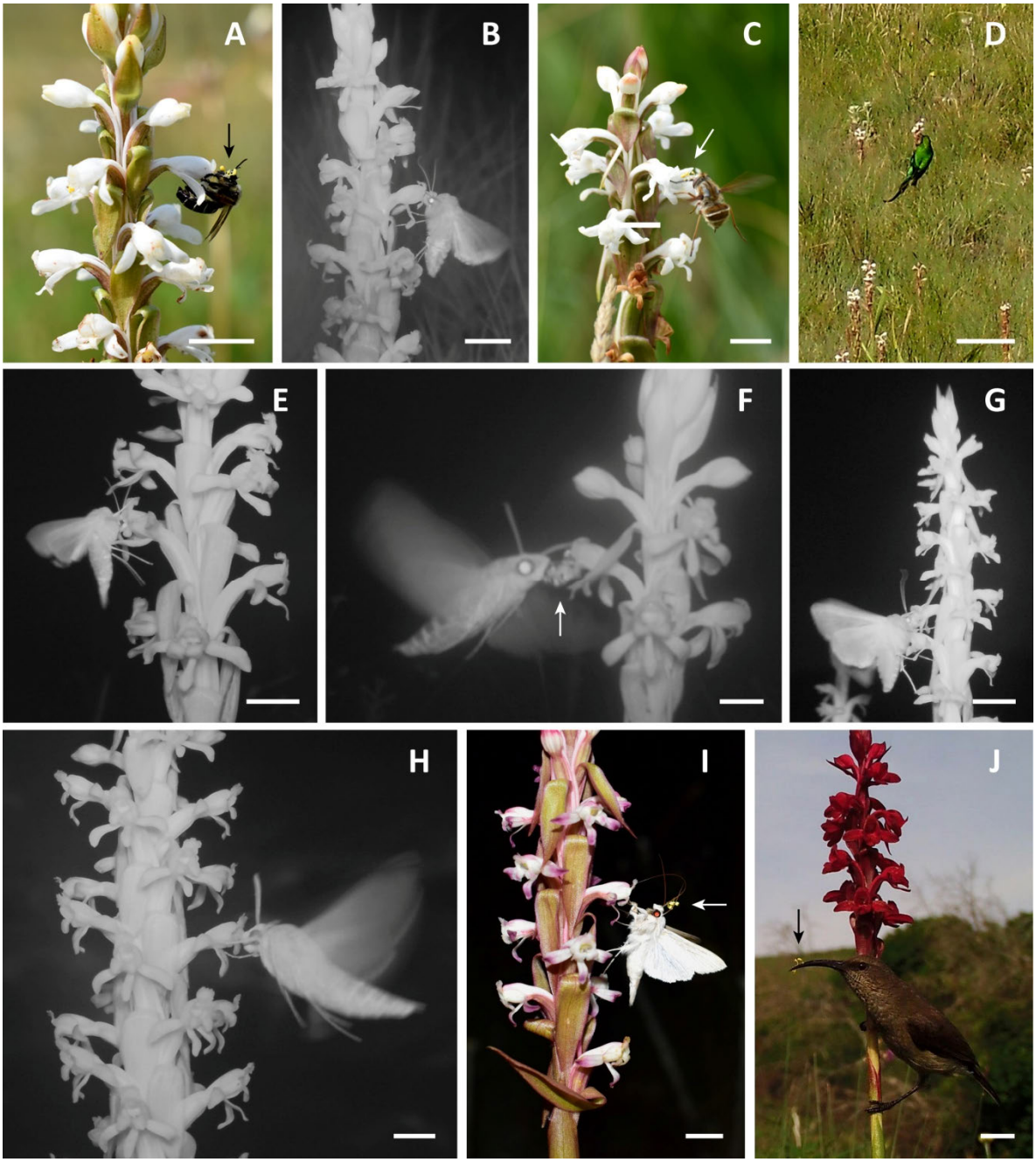

Figure 4.1. Morphotypes of the *Satyrium longicauda* species complex and their pollinators. A) *Rediviva neliana* removing pollinaria of OELB. B) *Thysanoplusia angulum* probing the spurs of OELM. C) OELM visited by *Prosoeca* sp. D) *Nectarinia famosa* visiting OELM. E) *Cucullia hutchinsoni* forcing its proboscis and head deep into a flower of OFL. F) *Hippotion osiris* depositing pollinaria on TELW. G) TFLD visited by *Thysanoplusia angulum*. H) *Basiothia schenki* visiting TSLG. I) *Cucullia hutchinsoni* visiting TFLP. J) *Chalcomitra amethystina* perched on *Satyrium rhodanthum*. Arrows show the presence of pollinaria on visitors. Pictures A, C, E, F, H show individuals at the Mt Gilboa site. Pictures B, D, G represent populations at the Tarn Cave site. Pictures I and J were taken at Kamberg and Jolivet respectively. Figures B, E-H, and J are screen grabs from videos obtained using motion-activated cameras. Scale bar = 10 mm except D = 10 cm.

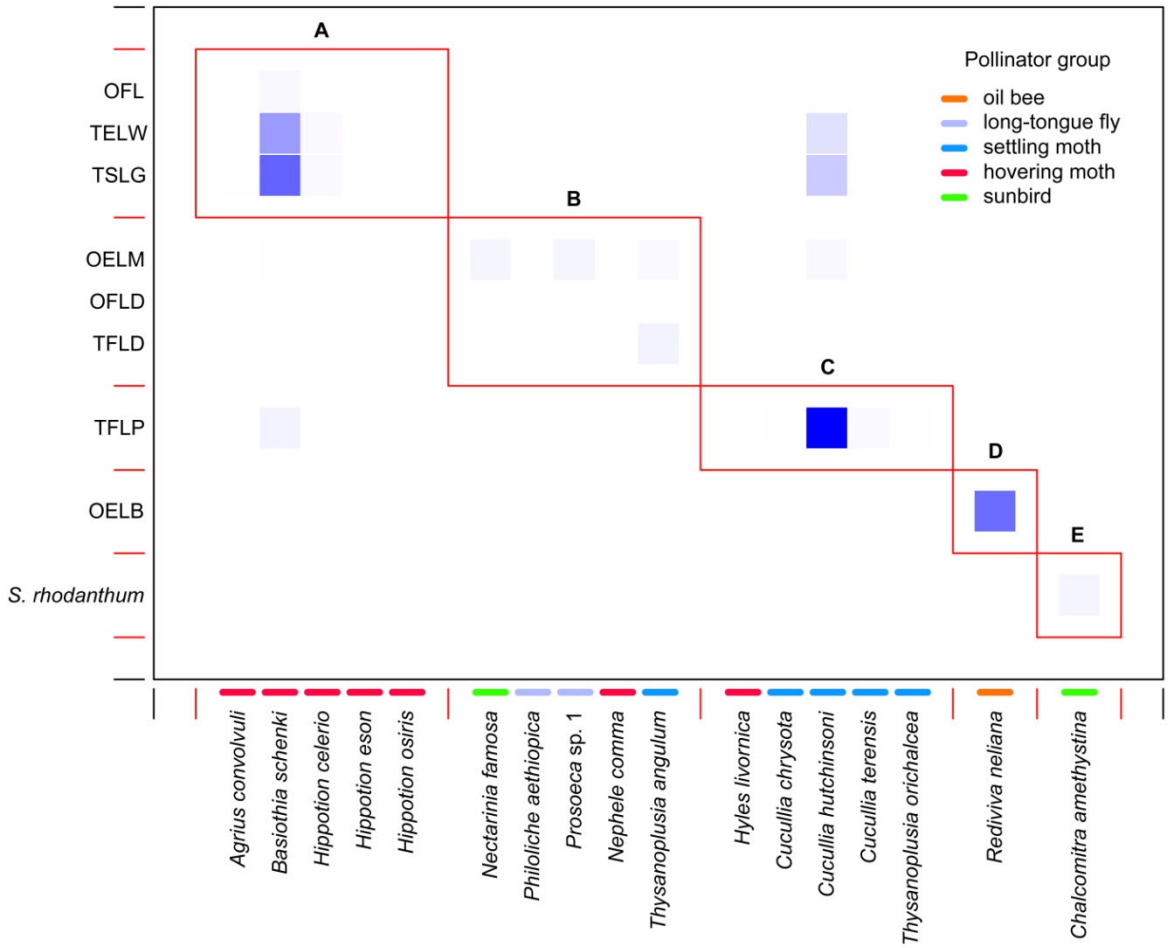

Figure 4.2. Modular structure of the plant-pollinator network between the morphotypes of the *S. longicauda* complex and their pollinators. Five modules (A-E) were identified using the DIRTLPAwb+ algorithm (Beckett 2016). Each module with morphotypes as rows and pollinators as columns is enclosed within a red edge. The frequency of the interactions is represented by different shades of the blue squares. The darker the blue colour, the higher the intensity of the interaction. Coloured bars indicate to which pollinator group the species belongs.

Association between modules and floral traits

Spur length

Functional spur length and pollinator proboscis length in populations representing seven morphotypes were strongly correlated (R^2 = 0.76, P = 0.001; Figure 4.3). The scatter plot reveals that populations of morphotypes that are part of module A clustered together and are clearly separate from morphotypes that belong to module C, whereas morphotypes within module B

showed a less clear separation from module C, consistent with the presence of extensive variation in the length of the spur and the tongue length of local pollinators.

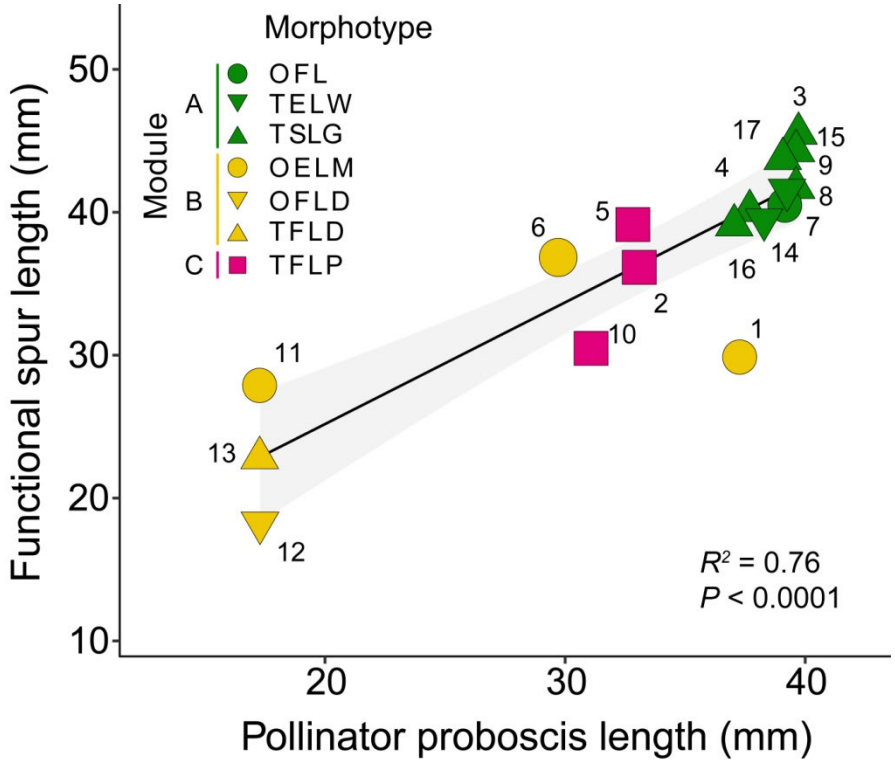

Figure 4.3. Correlation between pollinator proboscis length and functional spur length in populations of morphotypes of the *S. longicauda* complex (95% confidence interval shaded in grey). Key to populations: 1) Garden Castle. 2) Hlatikulu. 3) Jolivet. 4-5) Kamberg. 6-10) Mt Gilboa. 11-13) Tarn Cave. 14-15) Umtamvuna. 16) Verloren Valei. 17) Wahroonga.

Scent

Analyses of scent emission rates of populations sampled at day and night (N = 195) revealed significant effects of module, period and its interaction both at the level of flower (Figure 4.4A-4.4B; module: F = 217.61, P < 0.001; period: F = 85.95, P < 0.001; module × period: 171.69, P < 0.001) and inflorescence (Figure 4.4C-4.4D; module: F = 252.45, P < 0.001; period: F = 166.90, P < 0.001; module × period: 298.19, P < 0.001). Pairwise comparisons revealed that scent emission rates are significantly lower in Module E compared to all other modules (Figure 4.4A, 4.4C). Scent emission per flower between day and night (N = 195) sampling periods were similar except for module C and E (Figure 4.4B), whereas module A, C, and E differed when emission per

inflorescence was analysed (Figure 4.4D). Significant differences in emission among modules were observed both at the flower level (module: $F = 129.73$, $P < 0.001$) and inflorescence level (module: $F = 167.16$, $P < 0.001$; period: $F = 85.95$, $P < 0.001$) for the data set with night samples (N =205) only.

There was a significant difference in the number of compounds for modules, periods, and its interaction (Figure 4.4E-4.4F; module: $F = 16.13$, $P < 0.001$; period: $F = 11.45$, $P < 0.001$; module × period: 6.40, $P < 0.001$). Modules also differed in this for the data set with night samples only (module: $F = 10.89$, $P < 0.001$). A total of 109 floral volatiles were detected in the headspace samples of the nine morphotypes of *Satyrium longicauda* (Table S4.3). For populations for which samples were collected both during the day and night period, between 1 to 36 compounds were found, whereas for populations for which only night samples were obtained, the number of volatiles ranged from 0 to 30 per individual.

Overall, the scent of the morphotypes of *S. longicauda* was mainly dominated by terpenoids (monoterpenes and sesquiterpenes), followed by aromatics and esters (Table S4.3). Two discrete groups can be distinguished in the non-metric multidimensional scaling (NMDS) analysis, representing 1) all the morphotypes of *S. longicauda* and 2) *S. rhodanthum* (stress = 0.10; Figure 4.5A). Exclusion of the latter did not change the NMDS substantially, as the overlap among modules remained substantial, not only at night (stress = 0.15, Figure 4.5B) but also in populations for which both day and night samples were available (stress = 0.16).

A 2-way PERMANOVA of all volatiles present in 179 scent samples distributed across four of the five modules indicated that there were significant differences among the scent profiles of day and night samples at the period ($F = 24.37$, $p < 0.001$), and module ($F = 7.54$, $p < 0.001$) levels but not for their interaction ($F = -18.78$, $p < 0.83$). A SIMPER analysis showed that in all groups, regardless of time of day, benzenoid and phenyl propanoid volatiles such as (E)-cinnamyl alcohol, (Z)-cinnamyl alcohol, cinnamaldehyde, and benzaldehyde contribute the most to overall similarity within group (Table 4.1).

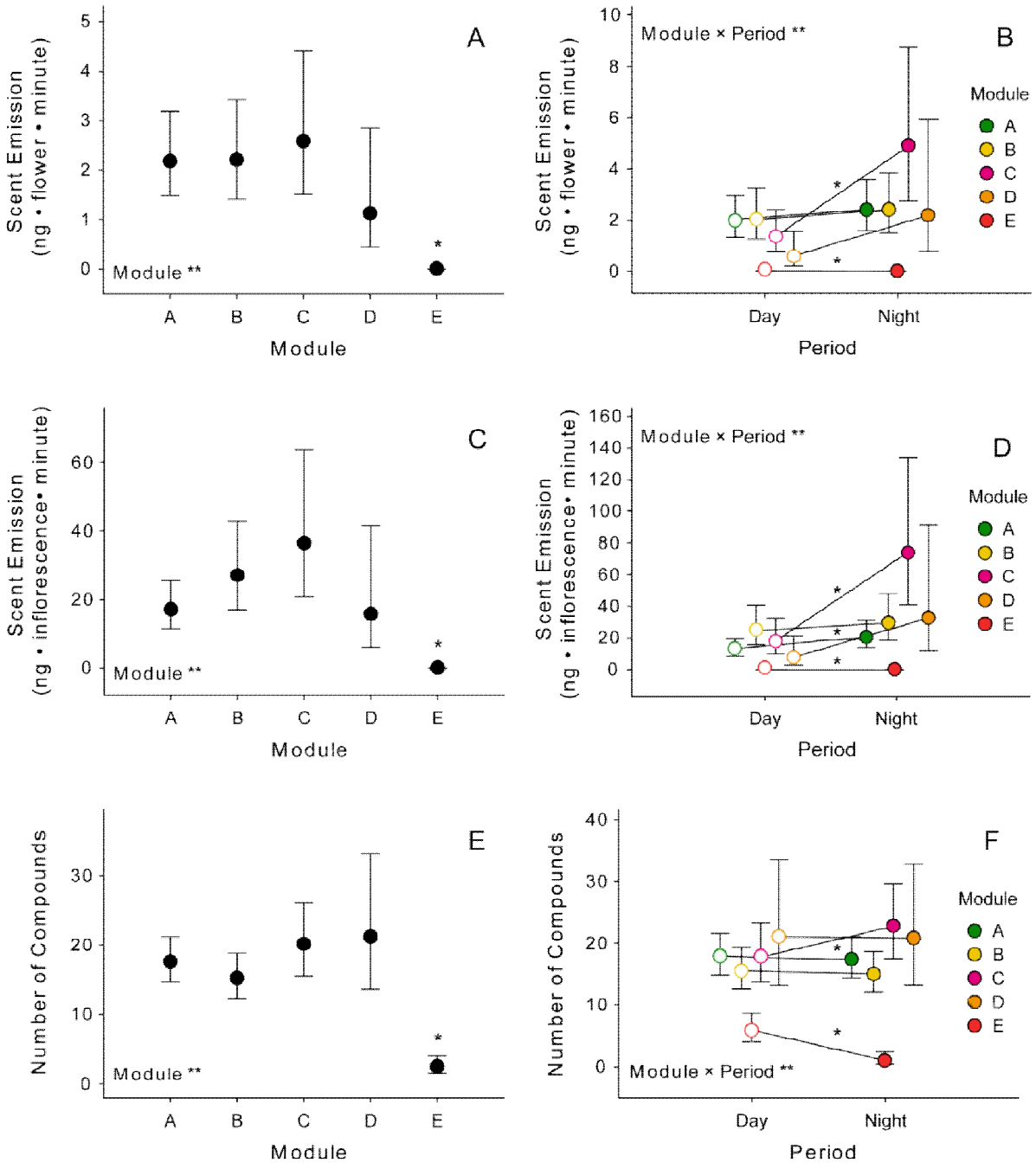

Figure 4.4. Effects of module on scent emission and number of scent compounds of *S. longicauda* day and night samples. A,C) Differences in the scent emission among modules per flower and inflorescence respectively. B,D) Differences of scent emission among modules between day and night period per flower and inflorescence respectively. E) Differences in the number of compounds among modules. F) Differences in the number of compounds among modules and between day and night period. Open and closed circles represent day and night sampling periods, respectively. Significances: ** ≤ 0.001. Significant differences in mean between pairs of modules are marked with an asterisk (p <0.05).

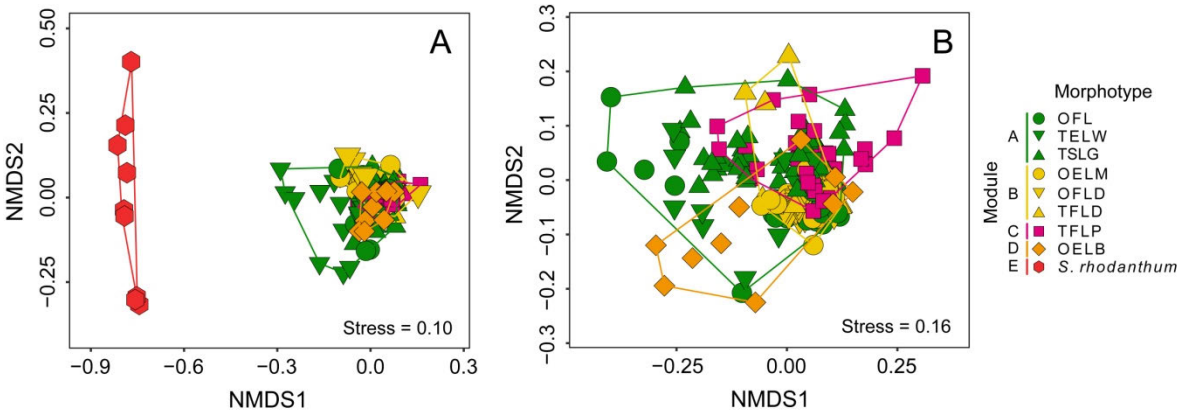

Figure 4.5. NMDS ordination of headspace scent samples for each module of the *S. longicauda complex based on the* Bray-Curtis similarity index. A) Differences in scent composition among 297 day-night samples of the five modules identified. Day and night samples have been included except for *S. rhodanthum* night samples. B) Variation in scent composition among 179 day-night samples distributed in the five modules. This analysis excluded *S. rhodanthum* since no scent was detected at night.

Colour

All five modules had weak reflectance in the UV region. Modules A, B, C, and D are characterised by white flowers, sometimes tinged with pink, and all showed an increase in reflectance between 360 and 400 nm, corresponding to the region of blue light, whereas module E exhibited a sharp increase in the green-red region around 590 nm (Figure 4.6A). The variability of spectral reflectance visualised in the colour space of Endler (1990) showed that most individuals fell in the third segment (S3; yellow-green) whereas only individuals of *S. rhodanthum* and four individuals of TFLD were plotted in the fourth segment (S4; red-blue) (Figure 4.6B).

The number of marker points among samples varied between zero and three. For 230 samples, one marker point was calculated, representing 88.46% of all samples, followed by 21 samples (8.1%) for which two and 2 (1.2%) samples for which three marker points were detected respectively. Only seven samples distributed among TFLP (5), OFLD (1) and TSLG (1) lacked marker points altogether. Primary marker points for samples within modules A and D were between 377 and 430 nm, whereas for modules B and C the primary marker point was observed between 382 and 661 nm. Module E had primary marker points between 620 and 641 nm. Wavelengths of the

primary marker point were significantly different among modules ($F = 47.15$, $p < 0.001$; Table 4.2) with module E values having higher values than all other modules (Tukey: $p < 0.05$). The GLM with binomial distribution indicated that the proportion of plants with single marker points did not differ significantly among modules ($\chi^2 = 8.84$, $P = 0.1$).

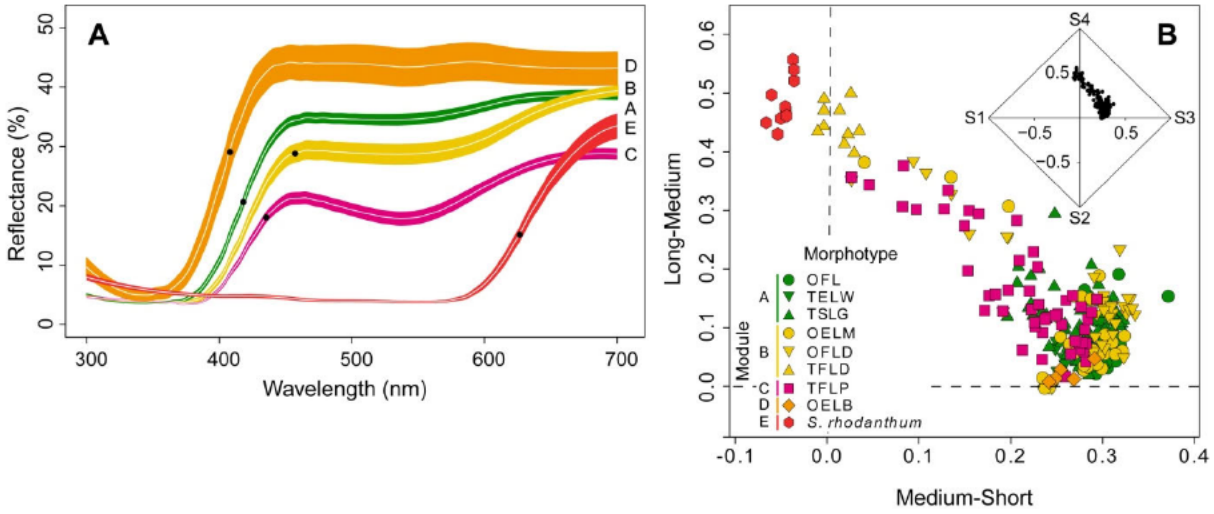

Figure 4.6. Lip colour variation in the *S. longicauda* complex. A) Mean colour spectral reflectance of modules. Areas represent ± SD. Mean wavelength of primary marker points are indicated as a black dot on the spectral reflectance. B) Variation in reflectance spectra across samples summarised using segment classification (Endler, 1990).

Table 4.2. Results of post-hoc Tukey comparing the wavelengths of the primary marker point

Module (N)	Primary Marker Point Wavelength (mean ± SE)*	Percentage of samples with one marker point
A (120)	413 ± 4.65 a	96.67
B (75)	457 ± 5.88 b	85.33
C (43)	433 ± 7.77 ab	81.40
D (5)	402 ± 22.79 ab	100
E (10)	634 ± 16.11 c	100

*Means with different letters differ significantly. $p < 0.05$

Table 4.1. Compounds contributing to the first 90% of average Bray-Curtis similarity within headspace samples of modules of the *S. longicauda* complex. Compounds in bold represent 70% of similarity within module.

Module	A				B				C				D			
Period	Day		Night		Day		Night		Day		Night		Day		Night	
Compound	%	sim/s.d.	%	sim/s.d.	%	sim/s.d.	%	sim/s.d.	%	sim/s.d.	%	sim/s.d.	%	sim/s.d.	%	sim/s.d.
Isobutyl butyrate	—	—	—	—	—	—	—	—	—	—	2.1	0.77	—	—	—	—
β-Pinene	1.3	0.52	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Styrene	4.04	0.98	3.95	1.02	—	—	—	—	**5.96**	1.57	3.51	1.54	3.7	4.52	**3.96**	1.98
Isoamyl butyrate	—	—	—	—	—	—	—	—	—	—	3.39	0.78	—	—	—	—
1-Hexanol	—	—	—	—	—	—	—	—	—	—	2.3	1.78	—	—	—	—
Benzaldehyde	**8.94**	2.87	**6.67**	1.87	**9.32**	3.33	**7.65**	2.37	5.31	3.17	**4.99**	2.73	**13.27**	3.05	**6.41**	3.69
Linalool	4.32	2.58	1.72	1.01	—	—	—	—	—	—	—	—	**5.28**	4.94	2.25	9.2
Lavandulol acetate	—	—	—	—	—	—	—	—	—	—	—	—	3.66	0.76	—	—
Caryophyllene	—	—	—	—	—	—	2.59	0.92	—	—	—	—	—	—	2.61	1.15
Lavandulol	—	—	—	—	—	—	—	—	—	—	—	—	1.79	0.59		
2-Tridecanone	—	—	—	—	—	—	—	—	—	—	—	—	—	—	2.25	5.1
6,10-Dimethyl-5,9-undecadien-2-one	—	—	2.32	1.78	2.36	2.9	—	—	2.5	3.29	—	—	—	—	—	—
Benzyl alcohol	5.24	3.1	**5.25**	4.08	3.92	4.28	4.68	5.23	4.23	4.89	3.6	5.32	**5.97**	6.24	**6.22**	2.63
(Z)-3-Phenylacrylaldehyde	3.09	2.49	—	—	2.63	4.86	—	—	2.33	7.84	—	—	3.9	7.33	—	—
Phenylethyl Alcohol	1.26	1.01	—	—	—	—	—	—	—	—	—	—	—	—	—	—
m/z: 133, 134, 115, 116, 105	—	—	—	—	—	—	—	—	2.13	4.07	—	—	—	—	—	—
2-Pentadecanone	—	—	—	—	—	—	—	—	—	—	—	—	**3.97**	3.06	3.58	3.27
Cinnamaldehyde	**9.18**	3.85	**10.5**	7.19	**9.99**	10.49	**10.8**	7.73	**9.94**	9.05	**10.3**	7.26	**6.47**	4.02	**8.63**	10.9

3-Phenylpropanol	3.42	1.15	4.91	1.78	4.43	11.96	5.21	8.66	**6.25**	8.14	**5.95**	7.46	3.09	2.91	**5.32**	10.93
Cinnamyl acetate	—	—	2.61	3.27	—	—	2.19	1.58	—	—	2.39	2.41	—	—	3.23	6.23
(Z-)-Cinnamyl alcohol	**15.7**	2.43	4.57	4.29	**13.1**	5.47	4.85	2.33	**10.16**	2.59	**4.23**	3.63	**17.24**	5.47	3.01	9.14
2-Heptadecanone	—	—	—	—	—	—	—	—	—	—	—	—	1.75	4.31	1.98	2.49
(E-)-Cinnamyl alcohol	**34.5**	3.33	**48.9**	5.9	**44.7**	15.08	**52.6**	7.18	**41.9**	5.61	**47.6**	5.94	**21.08**	4.04	**42.0**	4.59
Total	91	—	91.5	—	90.5	—	90.5	—	90.7	—	90.4	—	91.17	—	91.5	—
Average Bray-Curtis similarity	66.4	—	73.9	—	81.4	—	77.6	—	74.8	—	71.4	—	70.3	—	78.3	—
Number of compounds	11	—	10	—	8	—	8	—	10	—	11	—	13	—	13	—

% = percentage contribution of each compound to module similarity; sim/s.d. = percentage contribution/standard deviation. Compounds that characterise samples from a module will exhibit high percentage contributions and high sim/s.d. values.

Nectar

There were significant differences among modules in both nectar volume (Figure 4.7A; χ^2 = 878.215, p < 0.001) and concentration (Figure 4.7B; χ^2 = 138.712, p < 0.001). All modules differed in the volume of nectar, but module D showed the lowest value compared to other modules (Šidák's test, p < 0.001; Figure 4.7A). Significant differences were detected in nectar concentration between modules D and E, which were the modules with the lowest and highest sugar concentration respectively (sequential Šidák's test, p < 0.001; Figure 4.7B).

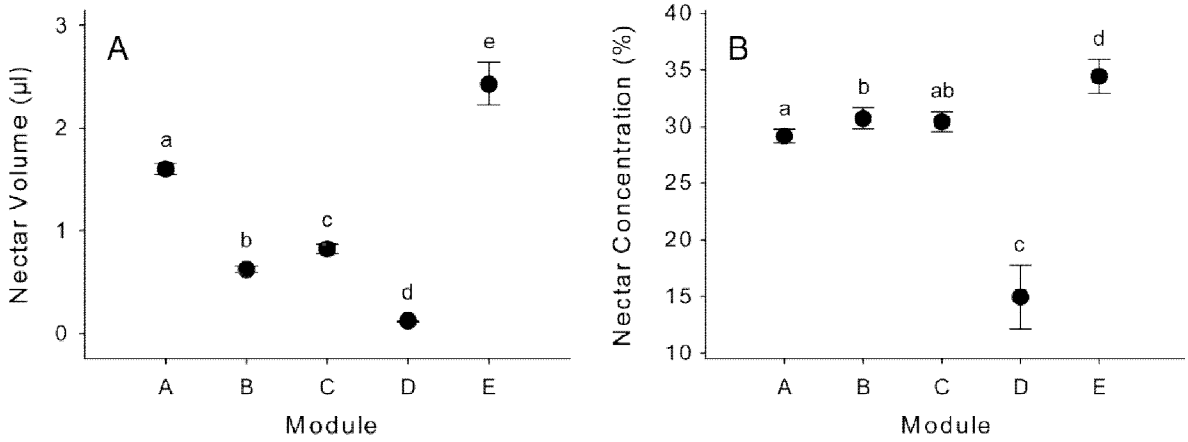

Figure 7. Nectar traits of *S. longicauda*. A) Variation in nectar volume among modules. B) Variation in nectar concentration among modules. Modules not sharing the same letter differ significantly. p < 0.001).

Discussion

The *Satyrium longicauda* plant-pollinator network is found to comprise five modules (Figure 4.2). Two of these potential pollination niches were dominated by moth pollination, one by oil-collecting bee pollination, one by sunbird pollination and one by a combination of long-tongued fly-, moth-, and sunbird pollination (Figure 4.2). The sunbird pollination module represented by *S. rhodanthum* is characterised by differences in scent, colour, and nectar from all other modules. Similarly, the oil-collecting bee pollination module differed markedly from the other modules in

traits measured here such as nectar, but also in the elevated presence of volatiles associated with production of oils (Castañeda-Zárate *et al.* 2021; Chapter 2). However, trait variation among the remaining modules which are to a greater or lesser degree characterised by moth pollination was less clear cut, apart from variation in spur length. Floral traits therefore differ among modules which are pollinated by different major functional pollinator groups, but most traits apart from spur length could not be distinguished among modules that differ more subtly in pollination systems.

Moth pollination in *S. longicauda*

Extensive pollinator observations carried out at a large number of sites broadened knowledge about the pollinator fauna of the highly variable *S. longicauda* (Table S4.2). The pollination systems of the two morphotypes belonging to the modules that are pollinated by sunbirds and oil-collecting bees respectively, have been described in detail elsewhere, and will not be discussed here (van der Niet *et al.* 2015; Chapter 2). The remaining modules were characterised by partial or exclusive pollination by moths, a scenario expected based on the morphological traits shared by most morphotypes included in these modules, such as scented white flowers with long spurs and the presence of nectar (Faegri and van der Pijl 1979; Vogel 2012). Indeed, moth pollination was already confirmed by several previous studies (Harder and Johnson 2005; Jersáková and Johnson 2007; Ellis and Johnson 2010; Johnson *et al.* 2011, 2019; Duffy and Johnson 2014; Castañeda-Zárate *et al.* 2021, Chapter 2).

All morphotypes in modules that are fully or partially moth-pollinated received visits by several species of Noctuidae and Sphingidae (see Table S4.2 for a full list of floral visitors). One of the modules, containing the three morphotypes OFL, TELW and TSLG, was characterised by hawkmoth pollination, in particular by *Basiothia schenki* (c.f. Harder and Johnson 2005; Jersáková and Johnson 2007; Ellis and Johnson 2010; Johnson *et al.* 2011; Duffy and Johnson 2014). In contrast another module, comprising three other morphotypes (TFLP, TFLD, and OFLD), was characterised by noctuid pollination. In particular, pollination of TFLP by *Cucullia hutchinsonii* was confirmed from several populations that differed from the ones surveyed in previous studies (Ellis and Johnson 2010; Johnson *et al.* 2011). Pollination by *Thysanoplusia angulum*, in the TFLD and OFLD morphotypes, is reported here for the first time.

The fifth module, which mostly comprised populations of the OELM morphotype, was characterised by bimodal pollination at daytime and at night. At Tarn Cave, OELM plants received visits by moths and malachite sunbirds (Figure 1B,D), whereas on Mt Gilboa the visitor spectrum included moths and long-tongued flies (Figure 1 C; Castañeda-Zárate *et al.* 2021; Chapter 2). The differences in diurnal visitation between these two sites cannot be explained by variation in pollinator distribution, since long-tongued flies are common at Tarn Cave, and malachite sunbirds are common at Mount Gilboa. Visits by sunbirds to OELM at Tarn Cave may be explained by a general lack of nectar sources for sunbirds at the time of year when *S. longicauda* flowers, whereas malachite sunbirds feed on several other plant species such as *Protea roupelliae* and *Disa chrysostachya* at Mt Gilboa during flowering of OELM (Hargreaves *et al.* 2004; Johnson and Brown 2004). The presence of pollination by sunbirds and long-tongued flies defied predictions based on particular floral signal preferences of these pollinator species. Sunbird-pollinated plant guilds typically include species with red to orange, unscented flowers that offer large amounts of dilute nectar in long corolla tubes or spurs (Johnson 1996; Johnson and Brown 2004; Waal *et al.* 2012; Hobbhahn and Johnson 2015; van der Niet *et al.* 2015; Johnson and van der Niet 2019). Species that are part of long-tongued fly pollination guilds are characterised by long corollas or spurs, that offer nectar as reward, lack scent, and can be either white, red, pink or blue-violet (Rebelo *et al.* 1985; Goldblatt and Manning 1999, 2000; Anderson and Johnson 2009; Woodcock *et al.* 2014; Newman and Johnson 2021). One interesting aspect about sunbird pollination in the OELM morphotype is that *S. rhodanthum* (a sunbird-pollinated member of the species complex) is closely related to the OELM morphotype (Chapter 3). The observation of sunbird visits to *S. longicauda* may therefore represent an intermediate stage of double function that was proposed by Stebbins (1970) as a pathway to explain transitions between pollinations systems.

Pollinator observations can be challenging if plants occur in remote areas, especially if they are visited at night. The use of motion-triggered cameras has contributed important data for quantifying the pollinator spectrum in this regard (Krauss *et al.* 2018; Johnson *et al.* 2019; Amorim *et al.* 2020; Tremlett *et al.* 2020). This was already known for documentation of vertebrate pollinators (Krauss *et al.* 2018; Wester 2019; Tremlett *et al.* 2020). Indeed, pollination by the sunbird *Chalcomitra amethystina* at Jolivet, a different population from the ones surveyed by van der Niet *et al*. (2015), was confirmed through both direct observations and the use of cameras. However, there is also growing evidence that motion-trigger cameras can be used successfully to

document insect pollination (Johnson *et al.* 2019; Castañeda-Zárate *et al.* 2021; Chapter 2). In this study, cameras confirmed visits to flowers by *Agrius convolvuli* and *Hippotion osiris*, which were seldom seen or never directly observed (Table S4.2; Figure 4.1F). Similarly, regular pollinators of OFL, such as *Basiothia schenkii* and *Cucullia hutchinsonii*, were mainly recorded using cameras, and the same applies to visits by relatively small moths such as *Thysanoplusia angulum* to OELM, OFLD and TFLD at Tarn Cave; a site where weather conditions like thick fog and wind make observations challenging. Future research should focus on the reliability of camera traps for recording visitation by relatively small insect species, under varying weather conditions, to assess whether they can be reliably used as a tool for quantifying insect visitation.

Floral traits and their association with pollination niches

The plant-pollinator network was highly modular. Strong niche partitioning was driven by a specialised group of pollinators that generally interacts only with a small number of morphotypes (Figure 4.2; Table S4.2; Ponisio *et al.* 2019). In particular, the interactions exemplified by modules D and E, which each contain only one morphotype pollinated exclusively by one pollinator species each, resulted in strong modularity. Although modules A and C are each highly specialised for moth pollination, they are connected through occasional sharing of two species: *Basiothia schenki* and *Cucullia hutchinsonii*. The main difference between the modules is due to the relative frequency of visits by these two pollinator species. The hawkmoth species *B. schenki* was the main pollinator of three morphotypes (OFL, TELW, and TSLG) within module A, whereas the noctuid species *C. hutchinsonii* was by far the most common pollinator of the TFLP morphotype which was therefore identified as a different module (C). The fact that both modules share at least one important pollinator may explain why the network is not perfectly modular. Module B is characterised by pollination by long-tongued flies, moths, and sunbirds and therefore has the widest niche breadth. In this study the use of a modularity analysis provided an objective way to characterise pollination niches and use these to investigate associations with variation in floral traits.

Relationship between pollinator mouthparts and spur length

Several studies have reported that tubular corollas or spurs of plants show a strong correlation with the length of the mouthparts of pollinators (Johnson and Steiner 1997; Alexandersson and Johnson 2002; Stang *et al.* 2009; Johnson *et al.* 2011). This association may be explained by the fact that trait matching influences the reproductive success of plants that rely on pollen vectors for reproduction (Nilsson 1988; Luyt and Johnson 2001; Muchhala and Thomson 2009; Pauw *et al.* 2009; Sletvold and Ågren 2010; Johnson *et al.* 2011; Kulbaba and Worley 2012, 2013; Kuriya *et al.* 2015). In *S. longicauda* functional spur length of modules with moth- or mixed pollination systems showed a high correlation with the mean proboscis length of pollinators (Figure 4.3). This result is somewhat surprising since pollinaria attach to the proboscis. In a system in which pollen are placed on the proboscis, as opposed to systems in which pollen is placed on the face or head, selection for trait matching between plant and pollinator may be weakened (Ellis and Johnson 2010). However, pollen placement on specific areas of pollinator proboscises may increase the likelihood that massulae are transferred to a conspecific stigma, and this may therefore explain the strong correlation. Indeed, spur length in *S. longicauda* was shown to be under selection through both male and female functions and this may lead to adaptation of spur length based on viscidium placement on optimal sites on the pollinator proboscis (Ellis and Johnson 2010).

Scent

Floral scent is a key signal in the attraction of many types of pollinators (Dobson 2006b). Several studies have shown that floral volatiles alone, or in combination with other floral traits determine the structure and intensity of plant-pollinator interactions in communities (Spaethe *et al.* 2007; Majetic *et al.* 2009; Yoshida *et al.* 2015), and differ among closely related species that differ in pollinator (Shuttleworth and Johnson 2010; Steenhuisen *et al.* 2010, 2012; Jürgens *et al.* 2013; van der Niet *et al.* 2014; Moré *et al.* 2021). Floral scent in *S. longicauda* varied in both the number of compounds and emission rates among the five modules (Figure 4.4). There were distinct differences in scent emission between the sunbird pollination module and the remaining modules. Sunbird pollination is characterised by both a lower number of compounds and lower scent emission. A low scent emission, or its complete absence, had previously been observed in other *Satyrium* species pollinated by sunbirds including *S. rhodanthum* (Johnson 1996; van der Niet *et al.*

2015; Johnson and van der Niet 2019). Similarly, emission rates and number of compounds in *Erica plukenetii* were also lower in sunbird- versus moth-pollinated ecotypes (van der Niet *et al.* 2014). A study comparing floral scent among species of *Protea* pollinated either by beetles or sunbirds showed that scent emitted from inflorescences of species pollinated by beetles was greater compared to species pollinated by sunbirds (Steenhuisen *et al.* 2012).

The number of compounds, as well as emission rates in the module formed by the OELB morphotype showed a larger variation in comparison with modules pollinated by nocturnal moths (Figure 4.4). However, both variables showed no significant differences to the remaining modules. The larger scent variation in the OELB morphotype may be a signature of weakened selection for maintenance of certain scent compounds based on the fact that the scent of the OELB morphotype may largely be vestigial, due to its recent shift away from moth-pollination (Castañeda-Zárate *et al.* 2021; Chapter 2). One caveat for understanding scent variation in *S. longicauda* is that I only measured volatiles from the floral headspace. However, a previous study showed that less volatile compounds do differ between OELB and moth-pollinated morphotypes of *S. longicauda* (Castañeda-Zárate *et al.* 2021; Chapter 2). In particular the difference in production of diacetin and presence of 2-tridecanone, thought to represent important compounds for oil-bee pollination (Schäffler *et al.* 2015), set the OELB morphotype apart from moth-pollinated morphotypes, although diacetin is present in small quantities in the moth-pollinated morphotypes (Castañeda-Zárate *et al.* 2021; Chapter 2). The presence of diacetin in the moth-pollinated modules may represent a pre-adaptation for oil-bee pollination (Castañeda-Zárate *et al.* 2021; Chapter 2). The presence of diacetin in other moth-pollinated South African orchids (Johnson *et al.* 2020) suggests that it may also function as moth attractant. More work is needed to evaluate whether, and to what degree, particular volatiles are functional. This should also consider less volatile compounds and take phylogenetic relationships into account, as these may provide an additional explanation for patterns of scent similarity (Steiner *et al.* 2011; Prieto-Benítez *et al.* 2016).

Differences in floral scent composition between phalaenophilous (settling moth-adapted) and sphingophilous (hawkmoth-adapted) species have been reported across different plant lineages (Knudsen and Tollsten 1993). Here, I did not find a distinction between modules pollinated primarily by settling moths and hawkmoths respectively (Figure 4.5). Both pollination niches, and

even the oil-collecting bee pollinated module, have scent blends with a similar composition. Benzenoids, aromatic compounds and monoterpenes dominated most blends (Table S4.3). In particular, (E)- and (Z)-cinnamyl alcohol, cinnamaldehyde, benzaldehyde, and benzyl alcohol accounted for at least 70% of the similarities among both sampling periods within modules (Table 4.1). Some of these floral volatiles have been found in other orchids specialised for moth pollination (Knudsen *et al.* 1993; Tollsten and Bergstrom 1993; Huber *et al.* 2005; Peter *et al.* 2009; Johnson *et al.* 2020) including *Satyrium* species that form part of a different clade from *S. longicauda* (van der Niet, Jürgens, *et al.* 2015). However, volatiles such as (E)- and (Z)-cinnamyl alcohol never dominated the scent of other *Satyrium* species and were sometimes completely absent from their blends. This may either reflect differences in the local pollinator fauna, or may be due to the fact that moths represent a niche that can be utilised through different functional traits.

The similarities observed in scent profiles among modules of *S. longicauda* regardless of the pollination agent is similar to a pattern previously reported in *Plantanthera bifolia* and *P. chlorantha* (Knudsen and Tollsten 1993). Although individuals of different populations of *P. bifolia* that differ in spur length were very variable in scent profiles, no significant differences between short- and long-spurred populations was detected, even though they are pollinated by different species of noctuid, geometrid and sphingid moths (Tollsten and Bergstrom 1993). On the other hand, ecotypes of the iris *Gladiolus longicollis* that differ in floral tube length and are pollinated by different hawkmoth species, have a distinct scent (Alexandersson and Johnson 2002; Anderson *et al.* 2010). Scent of the short-tubed ecotype pollinated by short-tongued hawkmoths is dominated by ocimene, benzyl acetate, phenylacetaldehyde, benzyl alcohol, and phenylethyl acetate, whereas linalool, and methyl benzoate dominated the long-tubed ecotype pollinated by long-tongued hawkmoths. A meta-analysis that focuses on comparison of scent in closely related species that are pollinated by different types of moths, as well as the use of electroantennographic detection and bioassays, may be useful ways of testing whether hawkmoths and noctuids select for different floral scent.

Colour

Flower colour variation in *S. longicauda* was partially associated with particular pollination niches. Using marker points as an objective way to analyse reflectance spectra and detect colour differences (Chittka and Menzel 1992; Dyer *et al.* 2012; Shrestha *et al.* 2013, 2019; Dorin *et al.* 2020), modules A, B and E were identified as distinct from each other (Table 4.2). The marker points of module E (around 600 nm; van der Niet *et al.* 2015) are particularly different from individuals from the remaining modules whose marker points were around 400 nm (Table 4.2). Although module A is significantly different from module B, the values observed in module C and D overlap with each of them. Therefore, the only clear separation of modules by a particular flower colour applies to the sunbird-pollination module versus the remaining modules (Figure 4.6). Similar to the analysis of marker points, individuals plotted in the Endler´s segment classification mostly showed continuous variation, except for *S. rhodanthum* (module E) and TFLD (part of module B) that occupied discrete clusters (Figure 4.6B). The reflectance spectrum of *S. rhodanthum* may be indicative of a process of convergent evolution for red colour in bird-pollinated flowers, as also been observed in Australian and Neotropical systems (Altshuler 2003; Burd *et al.* 2014). The red flower colour may not be necessary to attract birds, but may rather function to reduce conspicuousness to bees (Cronk and Ojeda 2008; Lunau *et al.* 2011; Chen *et al.* 2020). The observation that malachite sunbirds visited the white flowers of OELM (Figure 4.1D) could reflect the fact that birds do not show a strong preference for flower colour and that they can visit nectar-producing plant species that are temporarily abundant (Cronk and Ojeda 2008; Carlson and Holsinger 2021). Individuals within modules A to D that look white, white tinged with pink, or red to the human eye had mean primary marker point values between 400 and 460 nm consistent with moth pollination (Table 4.2; Figure 4.6). Although the module that is characterised by pollination by oil-collecting bees has a mean primary marker point wavelength very similar to that of individuals of the other modules apart from *S. rhodanthum*, the lack of visitation by nocturnal moths is most likely explained by the absence of nectar (Kelber *et al.* 2003; Stöckl and Kelber 2019). Other orchid species within the genera *Disperis* and *Pterygodium,* and even the congener *S. rhynchanthum,* characterised by their whitish colour are also pollinated by *Rediviva* bees (Pauw 2006; Steiner *et al.* 2011).

Nectar

Despite similarity in colour and scent, nectar volume differed among all modules (Figure 4.7A). Even though module C overlapped in concentration with modules A and B, each of the five pollination niches was characterised by a specific combination of nectar volume and concentration (Figure 4.7). Of these, the oil-collecting bee niche represents the most divergent one, because flowers of the OELB morphotype are in most cases devoid of nectar (Castañeda-Zárate *et al.* 2021; Chapter 2), and in case it was present in minute quantities it had a very low sugar concentration of around 15%. Conversely, plants belonging to the sunbird pollination niche had the highest mean nectar volume (2.47 µL) and sugar concentration (34.4%) of all niches. The mean nectar volume and concentration here observed are higher than the values reported by van der Niet *et al* (2015) for *S. rhodanthum* (possibly suggesting intraspecific variation in nectar properties) and other *Satyrium* species pollinated by sunbirds, such as *S. monadenum*, *S. neglectum* spp. *woodii*, and *S. sceptrum* (Johnson *et al.* 2011; Johnson and van der Niet 2019), but still fall within the range characteristic for sunbird pollination (Bartoš *et al.* 2012; van der Niet *et al.* 2014; Sun *et al.* 2017). The comparatively high sugar concentration for a bird-pollinated species may be a signature of a recent shift from moth pollination (Chapter 3). However, given that the sugar concentration in *S. rhodanthum* is higher than that of all other modules, it may also indicate selection by sunbirds for highly concentrated nectar. The nectar volume and concentration of members of the hawkmoth pollination niche (module A) is consistent with previous reports (Johnson *et al.* 2011). The members of the noctuid pollination niche (module C) had a lower volume compared to those of the hawkmoth pollination niche (0.82 µL), but a similar concentration (30.4%). The general association between nectar volume and spur length is consistent with other studies of moth-pollination systems (Martins and Johnson 2007, 2013) and also bee-pollination systems (Klumpers *et al.* 2019).

Pollination niches

The high modularity observed in the *S. longicauda* complex was reflected in the identification and composition of five modules. Floral trait overlap among modules occurred, but it is nevertheless possible to recognise distinct pollination niches. In particular, the sunbird pollination niche represented by module E (comprised of *S. rhodanthum*) is the most distinct among the five

modules and differs in scent, colour, and nectar properties. This may reflect the large differences between sunbirds and insect pollinators in terms of their sensory ecology and physiology. Although the oil-bee pollination niche is similar in scent properties and colour to modules partly or predominantly pollinated by nocturnal moths, the absence of nectar and presence of oil also renders it a highly distinct pollination niche (Castañeda-Zárate *et al.* 2021; Chapter 2).

Plants pollinated by moths are usually grouped into the phalaenophilous and sphingophilous syndrome respectively (Willmer 2011). Flowers pollinated by settling moths (phalaenophilous flowers) usually have a morphology that allows moth to land and walk on flowers. The flowers tend to be small and have short corolla tubes or spurs with small amounts of nectar. They are typically pale green or whitish in colour, and highly fragrant (Faegri and van der Pijl 1979). Conversely, sphingophilous flowers exhibit adaptations for large moths that forage by hovering in front of the flower rather than landing (Faegri and van der Pijl 1979; Vogel 2012). Such adaptations usually include flowers with long corolla tubes or spurs with a relatively large volume of nectar to accommodate the longer proboscides of hawkmoths (Martins and Johnson 2007, 2013; Johnson and Raguso 2016). The flowers are mostly pale or white in colour, and release a strong, heavy and sweet scent during anthesis which occurs at night (Knudsen and Tollsten 1993; Dobson 2006a). However, these combinations of floral traits are not always sufficient to separate each pollination syndrome, as most traits are shared with the exception of nectar volume, which is usually associated with floral tube length (Martins and Johnson 2007, 2013). Different studies have shown that moth-pollinated species are often not exclusively visited by one group of moth but rather by both groups (Goldblatt and Manning 2002; Peter *et al.* 2009; Johnson *et al.* 2011; Ortega-Baes *et al.* 2011; Tao *et al.* 2018; Lu *et al.* 2021). I found that patterns in modules that received exclusive or partial pollination by nocturnal moths showed substantial trait overlap in colour, scent, and nectar properties, regardless of whether the main pollinator group was noctuids (phalaenophily) or hawkmoths (sphingophily). The little divergence in floral traits found between modules characterised by pollination by settling moths and hawkmoths respectively may either indicate a lack of distinctiveness between both syndromes or a potential recent divergence (Raguso *et al.* 2006; Castañeda-Zárate *et al.* 2021; Chapter 2).

Trait matching between pollinator proboscis length and spur length may allow the separation of the two moth pollination niches (module A). Specifically, the length of the spurs of morphotypes in

module A are similar to the size of the tubular corollas or spurs of sphingophilous flowers generally (40-160 mm; Willmer 2011; Johnson and Raguso 2016). It is well established that phalaenophilous flowers tend to have short spurs compared to sphingophilous flowers (<25 mm; Faegri and van der Pijl 1979; Willmer 2011). However, modules of *S. longicauda* that received pollination by the noctuid *Cucullia hutchinsonii* with an unusually long (c. 30 mm) proboscis had longer spurs than plant species grouped within the phalaenophilous syndrome (Willmer 2011). Module B identified in this study is unusually broad, and includes various insects that are often associated with distinct specialised pollination systems in the southern African flora (long-tongued flies and moths; Goldblatt and Manning 2000; Johnson and Raguso 2016), as well as sunbirds. Mixed bird-insect pollination is also unusual (Waser and Price 1990; Mayfield *et al.* 2001; Devoto *et al.* 2006; Navarro-Pérez *et al.* 2017; Patrick *et al.* 2018). One possibility that can explain this result is that this module may contain several specialised modules, for instance in case different populations of the morphotypes included in module B are characterised by different specialised pollination systems. Although sunbirds were observed removing pollinaria, they may act mostly as nectar thieves if the relatively small viscidia fall off when birds move between plants. Nevertheless, sunbird visits to the OELM morphotype could represent an intermediate stage (Stebbins 1970) that facilitated the shift from moth to sunbird pollination in the closely related *S. rhodanthum* (Chapter 3). Further research on the function of variation in particular traits such as floral shape and orientation will be useful to understand the role of each pollinator group in driving selection, particularly in conjunction with experiments of pollination effectiveness such as single visit pollen deposition (King *et al.* 2013).

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Supplemental Information

Table S4.1. Study populations of the morphotypes of *Satyrium longicauda* and *Satyrium rhodanthum* and evaluated floral traits. Acronyms for morphotypes: OELB= One Erect Leaf-Bee; OELM= One Erect Leaf-Moth; OFL= One Flat Leaf; OFLD= One Flat Leaf-Dwarf; TELW= Two Erect Leaves-Wetland; TFLD= Two Flat Leaves-Dwarf; TSLG= Two Spreading Leaves-Giant; TFLP= Two Flat Leaves-Pink. Acronyms for site (provinces): EC= Eastern Cape; FS = Free State; NL= KwaZulu-Natal; LP= Limpopo; MP= Mpumalanga. Vouchers specimens of all populations were collected by M. Castañeda-Zárate and are housed in the Bews Herbarium (NU) at the University of KwaZulu-Natal.

Morphotype	Site	Representative voucher specimen	Colour	Nectar	Scent		Observations		Camera trapping	
					Day	Night	Day	Night	Day	Night
OELB	Mt Gilboa, NL	1731	5	61	5	5	66	18	180	180
OELM	Garden's Castle, NL	1683	—	—	—	—	—	4	—	—
	Kamberg, NL	1503	5	23	—	3	—	1	—	—
	Mt Gilboa, NL	1622	5	31	5	5	32	23	216	286
	Ngome, NL	1501	5	5	—	3	—	—	—	—
	Sani Pass, NL	1628	5	—	—	—	—	—	—	—
	Tarn Cave, NL	1630	5	16	5	5	9	9	72	72
	Umtamvuna, NL	1601	5	15	—	10	—	1	—	—
OFL	Highmoor, NL	1638	5	6	5	5	—	—	—	—
	Mpofana Rd, NL	1422	3	—	—	1	—	—	—	—
	Mt Gilboa, NL	1613	5	42	5	5	21	9	108	158
	Tarn Cave, NL	1631	5	18	5	5	5	2	—	—
	Verloren Valei, MP	1690	5	—	—	2	—	3	—	—
OFLD	Giant's Castle, NL	1623	5	17	5	5	—	—	—	—
	Highmoor, NL	1639	1	1	—	—	—	—	—	—
	Mount-Aux-Sources, NL	1434	5	8	—	3	—	—	—	—
	Sani Pass, NL	1421	5	8	—	2	—	—	—	—
	Sentinel Peak, NL	1616	5	9	—	5	6	1	—	—

	Tarn Cave, NL	1632	5	19	5	5	4	6	36	36
	Verloren Valei, MP	1430	5	3	—	3	—	—	—	—
	Witsieshoek, FS	1546	5	8	—	—	—	3	—	—
TELW	Mt Gilboa, NL	1635	5	32	5	5	14	29	144	234
	Red Desert, NL	1603	5	30	—	5				
	Umtamvuna, NL	1600	5	30	—	6	—	6	108	198
	Verloren Valei, MP	1427	5	—	—	—	—	2	—	—
TFLD	Sentinel Peak, NL	1436	5	10	—	3	—	1	—	—
	Tarn Cave, NL	1629	5	20	4	5	60	12	108	108
	Vrederus	—	—	—	—	—	—	1	—	—
TSLG	Bushman's Nek, NL	1536	5	15	—	3	1	—	—	—
	Coleford, NL	1656	5	18	5	5	—	—	—	—
	Highflats, NL	1497	5	15	—	3	2	—	—	—
	Highmoor, NL	1642	5	8	5	5	—	—	—	—
	Hogsback, EC	1566	5	5	—	3	—	—	—	—
	Jolivet, NL	1619	5	15	5	5	4	—	—	48
	Kamberg, NL	1552	5	12	—	10	—	6	—	—
	Mt Gilboa, NL	1634	5	31	5	5	17	24	108	258
	Ntsikeni, NL	—	—	—	—	—	—	2	—	—
	Priscila Valei, NL	—	5	5	—	3	—	1	—	—
	Queen Elizabeth Park, NL	1691	5	10	—	5	3	—	—	—
	Red Desert, NL	1602	5	30	—	5	—	—	—	—
	Umtamvuna, NL	1480	5	30	—	6	30	6	108	258
	Verloren Valei, MP	1428	5	5	—	—	—	1	—	—
	Vernon Crookes, NL	1615	5	16	5	5	2	—	—	—
	Wahroonga, NL	1532	5	15	—	—	—	—	—	—
	Woodbush, LP	1545	3	3	—	—	—	—	—	—
TFLP	Bushman's Nek, NL	1565	5	—	—	2	—	—	—	—
	Castleburn, NL	1584	5	15	—	—	—	—	—	—

	Coleford, NL	1651	5	10	5	5	1	—	—	—
	Highmoor, NL	1571	5	8	—	3	—	—	—	—
	Hlatikulu, NL	1585	5	15	—	5	1	—	—	—
	Inzinga, NL	1580	5	9	—	5	—	—	—	—
	Kamberg, NL	1569	5	20	—	6	21	18	78	232
	Lower Lotheni Rd, NL	1645	5	15	5	5	—	—	—	—
	Mt Gilboa, NL	1641	5	46	5	5	12	19	180	220
	uMkhomazi, NL	1577	3	5	—	3	—	—	—	—
	Highflats, NL	1498	5	15	3*	—	4	—	48	48
S. rhodanthum	Ixopo, NL	—	—	—	7*	—	—	—	—	—
	Jolivet, NL	1605	5	20	—	—	4	—	60	—

*Data taken from van der Niet et al., 2015.

Table S4.2. Visitors observed on Satyrium longicauda. Visitors are grouped by Family within their respective Order. Abbreviations of morphotypes of S. longicauda: OELB= one erect leaf-bee, OELM= one erect leaf-moth, OFL= one flat leaf, OFLD= one flat leaf-dwarf, TELW= two erect leaves-wetland, TFLD= two flat leaves-dwarf, TFLP= two flat ñeaves-pink, TSLG= two spreading leaves-giant. Proboscis length is given in milimeters. Representative vouchers were caught and prepared by Miguel Castañeda-Zárate unless stated otherwise.

Morphotype	Site	Species	Representative voucher	Observation hours (Direct/Camera)	Individuals recorded (direct observation/on camera/caught)	Individuals carrying pollinaria/individuals on which number of pollinaria could be recorded	Mean number of pollinaria (range)	Proboscis length (mean±SD)
OELB	Mt Gilboa	**HYMENOPTERA**						
		Melittidae						
		Rediviva neliana Cockerell, 1931	31		141 (137/4/16)	89/37	3.86 (1-12)	—
		Apidae						
		Xylocopa caffra (Linnaeus, 1767)	—		1 (1/0/0)	0	0	—
		LEPIDOPTERA						
		Pieridae						
		Colotis eris subsp. *eris* (Klug, 1829)	—	84/360	2 (1/1/0)	0	—	—
		Nymphalidae						
		Papilio nireus subsp. *lyaeus* Doubleday, 1845	—		1 (1/0/0)	0	—	—
		Noctuidae						
		Thysanoplusia ablusa (Felder & Rogenhofer, 1874)	32		1 (1/0/1)	0	0	17.21

OELM	Garden Castle	**LEPIDOPTERA**		4/0				
		Noctuidae						
		Cucullia hutchinsoni Hampson, 1902	—		4 (4/0/0)	2/0	—	—
		Unidentified	—		18 (18/0/0)	3/0	—	—
	Kamberg	**LEPIDOPTERA**		1/0				
		Noctuidae						
		Unidentified	—		2 (2/0/0)	0	—	—
	Mt Gilboa	**LEPIDOPTERA**		55/502				
		Sphingidae						
		Basiothia schenki (Möschler, 1872)	—		3 (0/3/0)	3/3	5 (4-6)	—
		Hippotion celerio (Linnaeus, 1758)	—		1 (0/1/0)	1/1	3	—
		Noctuidae						
		Cucullia hutchinsoni Hampson, 1902	89		3 (3/0/2)	2/2	5 (4-6)	32.82 ± 2.88
		Cucullia terensis Felder & Rogenhofer, 1874	130		1 (1/0/1)	0	0	18.43
		DIPTERA						
		Nemestrinidae						
		Prosoeca ganglbaueri Lichtwardt, 1910	118		1 (1/0/1)	0	0	21.20
		Prosoeca sp. 1	90		11 (11/0/2)	9/5	2.60 (1-4)	25.11 ± 2.41
		Tabanidae						
		Philoliche	91		1 (1/0/1)	1/1	1	15.8

		aethiopica (Thunberg, 1789)						
		HYMENOPTERA						
		Apidae						
		Amegilla natalensis Friese, 1922	94		3 (3/0/3)	0	0	13.82 ± 0.76
		LEPIDOPTERA						
		Sphingidae						
		Hippotion celerio (Linnaeus, 1758)	41		1 (1/0/1)	0	0	39.70
		Noctuidae						
	Tarn Cave	*Cucullia hutchinsoni* Hampson, 1902	—	18/144	2 (2/0/0)	0	—	—
		Thysanoplusia angulum (Guenée, 1852)	95		5 (3/2/1)	3/3	1.33 (1-2)	14.23
		Unidentified	—		8 (8/0/0)	0	—	—
		PASSERIFORMES						
		Nectariniidae						
		Nectarinia famosa (Linnaeus, 1766)	—		9 (9/0/0)	5/0	—	—
		LEPIDOPTERA						
		Sphingidae						
OFL	**Mt Gilboa**	*Basiothia schenki* (Möschler, 1872)	—	30/266	7 (4/3/0)	5/5	2.40 (1-3)	—
		Noctuidae						
		Cucullia hutchinsoni	—		2 (0/2/0)	1/1	1	—

		Hampson, 1902						
	Verloren Valei	**LEPIDOPTERA**						
		Sphingidae						
		Basiothia schenki (Möschler, 1872)	4	3/0	4 (4/0/1)	0	0	37.80
OFLD	**Tarn Cave**	**LEPIDOPTERA**						
		Noctuidae						
		Thysanoplusia angulum (Guenée, 1852)	98	4/36	3 (3/0/1)	1/1	1	16.52
		Thysanoplusia ablusa (Felder & Rogenhofer, 1874)	97		1 (1/0/1)	0	0	16.23
	Witsieshoek	**LEPIDOPTERA**						
		Noctuidae						
		Cucullia terensis Felder & Rogenhofer, 1874	119	2/0	1 (1/0/1)	0	0	26.71
		Cucullia sp.	—		1 (1/0/0)	0	—	—
TELW	**Mt Gilboa**	**LEPIDOPTERA**						
		Sphingidae						
		Basiothia schenki (Möschler, 1872)	42	43/378	94 (87/7/11)	32/18	4 (1-10)	41.67 ± 2.00
		Hippotion celerio (Linnaeus, 1758)	—		3 (2/1/1)	2/2	7 (4-10)	42.1
		Hippotion osiris (Dalman, 1823)	—		1 (0/1/0)	1/1	10	—
		Unidentified	—		9 (9/0/0)	4/0	—	—
		Noctuidae						

		Cucullia hutchinsoni Hampson, 1902	102		30 (22/8/8)	14/10	2.80 (1-6)	32.42 ± 1.26
		Cucullia terensis Felder & Rogenhofer, 1874	RJC-202		1 (1/0/1)	1/1	1	26.92
		Pieridae						
		Belenois aurota subsp. *aurota* (Fabricius, 1793)	88		1 (1/0/1)	0	0	8.04
		DIPTERA						
		Nemestrinidae						
		Prosoeca sp. 1	101		5 (5/0/4)	0	0	26.53 ± 2.87
		LEPIDOPTERA						
		Sphingidae						
		Basiothia schenki (Möschler, 1872)	—		2 (2/0/0)	1/1	—	—
	Umtamvuna	*Hippotion celerio* (Linnaeus, 1758)	27	6/108	3 (3/0/1)	1/1	7	36.81
		Hippotion eson (Cramer, 1779)	28		1 (1/0/1)	1/1	4	42.80
		Agrius convolvuli (Linnaeus, 1758)	—		2 (0/2/0)	1/1	2	—
		LEPIDOPTERA						
	Verloren Valei	Sphingidae		2/0				
		Unidentified	—		2 (2/0/0)	0	—	—
		HYMENOPTERA						
TFLD	**Tarn Cave**	Apidae		66/108				
		Amegilla natalensis Friese,	56		31 (31/0/10)	0	0	13.44 ± 0.99

		1922					
		LEPIDOPTERA					
		Hesperiidae					
		Unidentified	57		1 (1/0/1)	0	0	14.79
		Noctuidae					
		Cornutiplusia circumflexa (Linnaeus, 1767)	—		2 (2/0/0)	0	—	—
		Athetis cf. *hexencauma* Krüger, 2005	69		7 (7/0/2)	0	0	7.77 ± 0.59
		Hadena bulgeri (Felder & Rogenhofer, 1874)	71		3 (3/0/1)	0	0	7.97
		Thysanoplusia angulum (Guenée, 1852)	95		12 (7/5/2)	5/5	1.6 (1-2)	19.22 ± 0.65
		DIPTERA					
		Nemestrinidae					
		Prosoeca sp. 2	59		34 (34/0/24)	0	0	18.16 ± 1.20
		LEPIDOPTERA					
		Noctuidae					
	Vrederus	*Cucullia hutchinsoni* Hampson, 1902	—	1/0	1 (1/0/0)	0	0	—
		LEPIDOPTERA					
		Nymphalidae					
TSLG	**Bushman's Nek**	*Danaus chrysippus* subsp. *orientis*	2	1/0	1 (1/0/1)	0	0	12.60

	(Aurivillius, 1909)						
Jolivet	**LEPIDOPTERA**		4/48				
	Sphingidae						
	Basiothia schenki (Möschler, 1872)			1 (0/1/0)	1/1	10	—
Ntsikeni	**LEPIDOPTERA**		2/0				
	Sphingidae						
	Basiothia schenki (Möschler, 1872)	125		2 (2/0/2)	1/1	9	40.57 ± 1.77
	Unidentified	—		3 (3/0/0)	1	—	—
	Noctuidae						
	Cucullia hutchinsoni Hampson, 1902	121		9 (9/0/5)	2/2	1.50 (1-2)	31.50 ± 0.65
	Thysanoplusia angulum (Guenée, 1852)	126		1 (1/0/1)	0	0	15.11
Mt Gilboa	**LEPIDOPTERA**		41/366				
	Sphingidae						
	Agrius convolvuli (Linnaeus, 1758)	49		2 (2/0/1)	1/1	1	91.22
	Basiothia schenki (Möschler, 1872)	47		88 (65/23/5)	48/22	5.1 (1-10)	41.08 ± 2.63
	Noctuidae						
	Cucullia hutchinsoni Hampson, 1902	—		31 (20/11/0)	19/9	3 (1-6)	—
Kamberg	**LEPIDOPTERA**		6/0				
	Sphingidae						
	Basiothia schenki (Möschler, 1872)	51		7 (7/0/2)	6/2	1.50 (1-2)	43.42 ± 1.36

		Noctuidae						
		Cucullia hutchinsoni Hampson, 1902	55		9 (9/0/4)	4/2	1.50 (1-2)	34.01 ± 0.55
		LEPIDOPTERA						
		Sphingidae						
	Umtamvuna	*Basiothia schenki* (Möschler, 1872)	29	36/366	6 (5/1/2)	3/2	5.50 (1-10)	39.51 ± 2.86
		Hippotion osiris (Dalman, 1823)	—		1 (0/1/0)	0	—	—
		LEPIDOPTERA						
	Verloren Valei	Sphingidae		1/0				
		Basiothia schenki (Möschler, 1872)	3		1 (1/0/1)	1/1	1	36.50
		LEPIDOPTERA						
	Coleford	Noctuidae		1/0				
		Cucullia hutchinsoni Hampson, 1902	116		1 (1/0/1)	0	0	33.92
		LEPIDOPTERA						
		Sphingidae						
		Basiothia schenki (Möschler, 1872)	—		9 (9/0/0)	4/0	—	—
TFLP	**Kamberg**	Unidentified	—	39/310	2 (2/0/0)	0	—	—
		Noctuidae						
		Cucullia hutchinsoni Hampson, 1902	128		141 (120/21/7)	84/12	3.08 (1-9)	32.51 ± 1.34
		Unidentified	—		9 (9/0/0)	0	—	—
	Mt Gilboa	**LEPIDOPTERA**		31/400				
		Sphingidae						

		Basiothia schenki (Möschler, 1872)	—		1 (1/0/0)	0	—	—
		Hippotion eson (Cramer, 1779)	25		1 (1/0/1)	0	—	45.08
		Hyles livornica (Esper, 1780)	19		1 (1/1/1)	1/1	1	28.81
		Noctuidae						
		Cucullia chrysota Hampson, 1902	26		2 (2/0/2)	1/1	2	25.58 ± 1.44
		Cucullia hutchinsoni Hampson, 1902	14		84 (70/14/13)	68/9	2.67 (1-4)	32.01 ± 1.39
		Cucullia terensis Felder & Rogenhofer, 1874	21		5 (5/0/2)	2/2	2.50 (2-3)	28.01 ± 0.71
		Thysanoplusia orichalcea (Fabricius, 1775)	22		3 (3/0/2)	1/1	1	16.66 ± 1.16
		Thysanoplusia ablusa (Felder & Rogenhofer, 1874)	20		1 (1/0/1)	0	0	17.41
		Ochropleura perirrorata (Hampson, 1902)	76		1 (1/0/1)	0	0	7.21
		PASSERIFORMES						
		Nectariniidae						
S. rhodanthum	**Highflats**	*Chalcomitra amethystina* (Shaw, 1812)*	—	4/96	9 (—/—/0)	5/0	—	—
	Jolivet	**PASSERIFORMES**		4/60				

	Nectariniidae *Chalcomitra amethystina* (Shaw, 1812)	—		2 (1/1/0)	1/1	3	—

*Data taken from van der Niet *et al.*, 2015.

CHAPTER 5

Satyrium longicauda var. *redivivense*: A NEW VARIETY IN THE *Satyrium longicauda* COMPLEX (ORCHIDACEAE) FROM SOUTH AFRICA

Abstract

Satyrium longicauda var. *redivivense*, from the Midlands area of KwaZulu-Natal, South Africa, is described and illustrated. The new variety can be diagnosed by the combination of a single elliptic to lanceolate erect leaf, distinctly downward-facing flowers with spreading sepals, relatively short spurs that lack nectar, and production of elevated levels of diacetin. The new variety is exclusively pollinated diurnally by the oil-collecting bee species *Rediviva neliana.* Due to the continuous nature of variation in diagnostic morphological traits within *S. longicauda*, the new taxon is recognised as variety rather than as a separate species from *S. longicauda*. The formal recognition of the new variety, which is known from two adjacent populations, makes it of immediate conservation concern.

Key words: Critically Endangered, grassland, KwaZulu-Natal, oil-collecting bee, *Reviviva*, *Satyrium*

Introduction

Satyrium Sw. comprises about 100 species of terrestrial orchids that are distributed mainly in southern Africa, with a few species in eastern and western Africa, Madagascar, and four species in Asia (Hall, 1982; Kurzweil and Linder, 2001, 1999; van der Niet, 2017; van der Niet et al., 2011b, 2009). Flowers with a helmet-like labellum from which two spurs project characterize the genus (Hall, 1982; Kurzweil and Linder, 2001, 1999). The diverse floral morphology present in the genus has been demonstrated to be the result of adaptation to different pollinators (Johnson, 1997a, 1997b; Johnson et al., 2011, 2007; van der Niet et al., 2015, 2011a). Within the genus, several species, including *S. longicauda, S. neglectum*, *S. nepalense*, and *S. sacculatum*, can be considered species complexes that exhibit various stages of diversification among clusters of related forms (Kurzweil and Linder, 1999). Of these, *S. longicauda* has a particularly wide geographical distribution and two varieties are currently recognized (Hall, 1982).

Recent fieldwork in the Midlands area of KwaZulu-Natal, South Africa, specifically in the Mt Gilboa Nature Reserve, showed the presence of six distinct morphotypes within the *S. longicauda* complex (Castañeda-Zárate et al., 2021; Chapter 2). Five of these morphotypes are pollinated nocturnally by nectar-feeding moths, whereas one is exclusively pollinated diurnally by oil-collecting bees (Castañeda-Zárate et al., 2021; Chapter 2). A phylogenetic analysis, including multiple accessions of each morphotype, showed that the individuals of the morphotype pollinated by oil-collecting bees comprise a monophyletic clade (Castañeda-Zárate et al., 2021; Chapter 2). Together, this evidence suggests that the presence of different pollination systems underpins morphological divergence and maintains reproductive isolation among *S. longicauda* morphotypes (Castañeda-Zárate et al., 2021; Chapter 2), and thus conserves species integrity between the morphotypes that are pollinated diurnally and nocturnally (cf. Grant 1994). Therefore, available evidence supports the recognition of the form pollinated by oil-collecting bees as a distinct taxon. The aim of this study is to formally describe a new variety, thereby separating it from the widespread and common varieties of *S. longicauda* and emphasizing its novel conservation status.

Material and Methods

The taxonomic description was based on fresh material collected during fieldwork carried out between 2016 and 2020 in the Mt Gilboa Nature Reserve and adjacent areas (the exact locality is withheld here to prevent illegal collecting). Collected specimens were pressed and dried and flowers were preserved in 70% ethanol with a few drops of glycerol. Measurements were obtained from fresh material and spirit-preserved flowers. Flowers were dissected and observed under an Axio Lab.A1 Zeiss stereomicroscope. Vegetative characters were measured to the nearest 1 mm using a ruler, whereas floral characters were measured to the nearest 0.01 mm with a pair of digital callipers. A line drawing and Lankester composite dissection plate (LCDP) were prepared based on photographs taken with a Nikon D5600 digital camera, and an Axio Lab.A1 Zeiss stereomicroscope. Morphological characters were described using terminology following Beentje (2010). Other *S. longicauda* specimens (including types) from the Bews Herbarium (NU) and the South African National Biodiversity Institute (PRE) were examined, but none of them included specimens of the new variety. Voucher specimens of the new variety (including holotype and isotypes) were lodged at NU, BOL, PRE, and NBG (herbarium acronyms according to Thiers, 2020).

The conservation status of the new variety was assessed following the system presented in the IUCN Red List Categories and Criteria: Version 3.1 (IUCN, 2012), and revised guidelines (IUCN Standards and Petitions Committee, 2019).

Results and Discussion

Satyrium longicauda Lindl. var. *redivivense* Castañeda-Zárate & van der Niet var. nov. (Figures 5.1, 5.2)

Satyrium longicauda var. *redivivense* differs from var. *longicauda* and var. *jacottetianum* by having smaller and nodding flowers with sepals less reflexed and more spreading, a slightly curved ovary, spurs that lack nectar, and high levels of diacetin.

Type: South Africa. KwaZulu-Natal, Albert Falls (2930): uMgungundlovu district, Mpofana municipality, Mt Gilboa Nature Reserve (-AD), in Drakensberg Foothill Moist Grassland, alt. 1603 m, 21 Dec 2020, M. Castañeda-Zárate MCZ-1760 (HOLOTYPE: NU; ISOTYPES: NU, BOL, PRE, NBG).

Description: Terrestrial herb. **Roots** few, glabrous, 31–58 mm long, 0.7–2 mm in diameter. **Tubers**, the mature slightly wrinkled, ellipsoid to obovoid, 20.2–28.2 × 8–14.2 mm, the young turgid round, 5.6–13 × 6.4–12 mm. **Reproductive** above-ground **stem** 210–490 mm long including the inflorescence × 2.3–5.7 mm diameter, with 6–8 sheathing leaves clasping the stem, 23.8–77.7 × 5.3–24 mm. **Leaf** one, erect, basal, born on a sterile shoot next to the reproductive stem, lanceolate to elliptic, entire, acute at apex, hyaline margin, 50–154 × 23–57 mm, with 6–1 parallel hyaline veins; **stem** 16.5–56 mm x 3–5 mm in diameter. **Inflorescence** a raceme, glabrous, 45–183 mm long, 2.1–5.03 mm diameter, with 7–49 densely arranged flowers; floral bracts reflexed at anthesis, 10.1–32.1 × 3.2–10.7 mm, lanceolate, acute to attenuate, 3–7 veined, glabrous, hyaline-reddish margin, minutely and sparsely ciliate. **Flowers** non-resupinate, white, fleshy, with a nodding appearance, spur tips and base of sepals and lateral petals green; sweetly scented. **Ovary** slightly arched, rarely abruptly bent downwards, 6–12 × 2.1–5 × 1.5–4 mm, longitudinally ridged, green with light red-brown tinges mostly on the ribs, standing away at an acute angle from rachis. **Labellum** 2–spurred, galeate, globose, with a weakly developed dorsal ridge, margins reflexed, apex broadly obtuse; apical **flap** prominent, rounded to emarginated, 1.2–2.0 mm high and 1.6–3 mm wide, erect to recurved, unevenly repand to sinuate, inner surface hairy; side of labellum forming a tube-like structure with petals; base of sepals and petals sparsely hairy inside, sometimes petals with sparse hairs not only at the base but along their entire length; galea 6.6–8.7 mm × 3.5–5 mm, hairy inside; galea aperture 1.3–2.8 mm high and 1.7–3.4 mm wide, rarely forward-facing, mostly slightly to markedly downwards facing. **Spurs** 6.6–24.1 mm long, 0.8–1.1 mm in diameter, parallel to ovary. **Sepals** and **median petals** fused for 1.2–2.3 mm. Free part of **lateral sepals** oblong-elliptic, with narrow rounded to obtuse apex, rarely acute, entire, projecting forwards for about half of their length, then gradually recurving sideways or rarely reflexed, 6.8–10.1 mm × 1.8–4 mm. Free part of **median sepal** oblong, rounded to obtuse, entire, projecting forward fully or ¾ of its length, then weakly projecting downwards, 6.4–10.6 mm × 1.5–2.5 mm. Free part of **lateral petals** oblong, rounded to obtuse, entire, projecting forward for ¾ of their length, then gradually to abruptly recurving downwards, 6.3–8.9 mm × 1.6–2.5 mm. **Gynostemium** filling back of galea. **Column** part slightly or rarely abruptly bent towards apex, 4.1–5.7 mm × 0.5–1

mm, white. **Anther** reflexed and aligned with column, 1.2–1.5 × 0.6–1 mm, connective broadly retuse, not extending beyond individual anther thecae**. Pollinaria** 2, obovoid, each with 1 sectile pollinium joined to the viscidium by a slender caudicle. **Stigma** erect, truncate, emarginate, 1–1.6 mm × 1.2–2.5 mm. **Rostellum** triangular, 1.2–2 mm × 1.3–1.9 mm; rostellum apex narrowly spoon-shaped; **viscidia** lateral, elliptic, 0.4–0.6 × 0.3–0.5 mm, plate like. **Lateral anther appendages** placed at anther base, 0.2–0.5 mm in diameter. **Capsule** ellipsoid-falcate, 6.8–9.3 × 2.7–3.8 × 1.7–2.7 mm, trigonous, 3-carinate, with the rest of perianth at the apex, green to reddish. **Seeds** fusiform, 0.20–0.38 × 0.09–0.14 mm; embryo prolate spheroid, 0.08–0.22 × 0.06–0.10 mm.

Distribution and habitat: *Satyrium longicauda* var. *redivivense* is only known from two populations located within two adjacent Nature Reserves, Mt Gilboa and Karkloof from 1602 to 1624 m, in Drakensberg Foothill Moist Grassland (cf. Mucina and Rutherford, 2006). Plant species recorded in the population found within the Mt Gilboa Reserve that grow intermingled with *Satyrium longicauda* var. *redivivense* include other herbs, forbs and grasses such as: *Ajuga ophrydis*, *Berkheya speciosa*, *Brungsvigia undulata*, *Corycium* sp., *Disa stachyoides*, *D. versicolor*, *Disperis cardiophora*, *Eragrostis* sp., *Eriosema distinctum*, *Helichrysum* spp., *Lotonotis eriocarpa*, *Moraea inclinata*, *Sebaea sedoides*, *Senecio* spp., *Silene burchellii*, *Stachys natalensis*, and *Themeda triandra*.

Ecology and phenology: *Satyrium longicauda* var. *redivivense*, like other grassland species, is highly dependent on fire for growing/sprouting and flowering. Burning of the grasslands in the summer rainfall area takes place during the dormant season that lasts from May to September before the first spring rains. Flowering occurs mainly in December with late individuals also blooming in early January. In culture, plants have been observed flowering in November. Fruits open and release their seeds approximately four weeks after pollination.

Pollination: Females of the oil-collecting bee species *Rediviva neliana* exclusively pollinate the flowers of *Satyrium longicauda* var. *redivivense*. Pollinaria get attached to the tibia of their forelegs while rubbing the oil-secreting trichomes present in the inner surface of the labellum (Castañeda-Zárate et al., 2021; Chapter 2).

Conservation status: Both only known populations and subpopulations are nestled within two adjacent Nature Reserves and are therefore protected. Nonetheless, due to its limited area of occupancy (AOO), which is estimated ≤ 1 km^2, its conservation status according to the IUCN Red List Categories and Criteria (IUCN, 2012; IUCN Standards and Petitions Committee, 2019) is Critically Endangered (CR), criterion B2a.

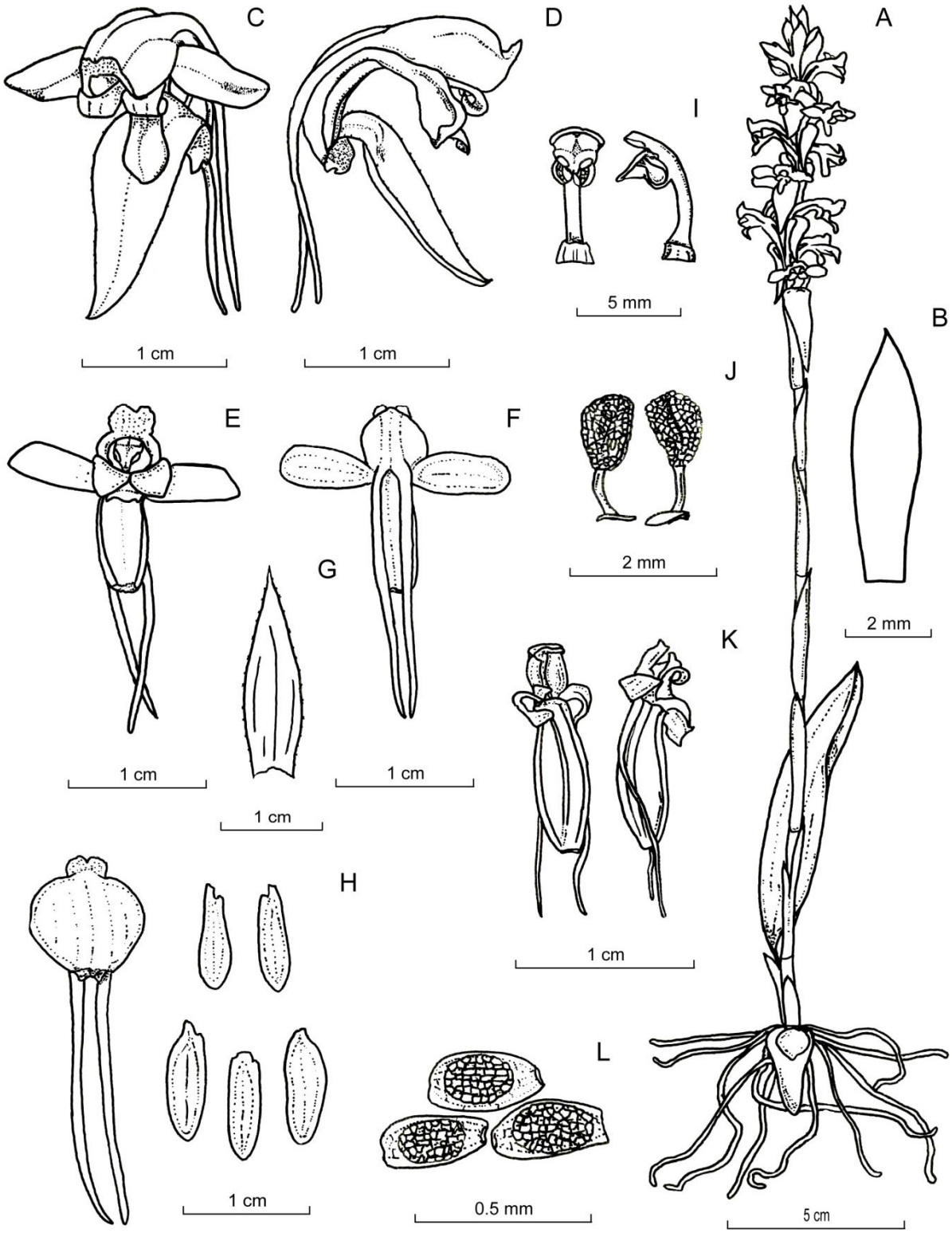

Figure 5.1. *Satyrium longicauda* var. *redivivense* Castañeda-Zárate & van der Niet. (a) Habit. B. Leaf sheath. C. Flower oblique view. D. Flower side view. E. Flowers frontal view. F. Flower dorsal view. G. Floral bract. H. Dissected perianth. I. Column in ventral and lateral view. J. Pollinaria. K. Capsule in frontal and lateral view. L. Seeds. Drawn by M. Castañeda-Zárate.

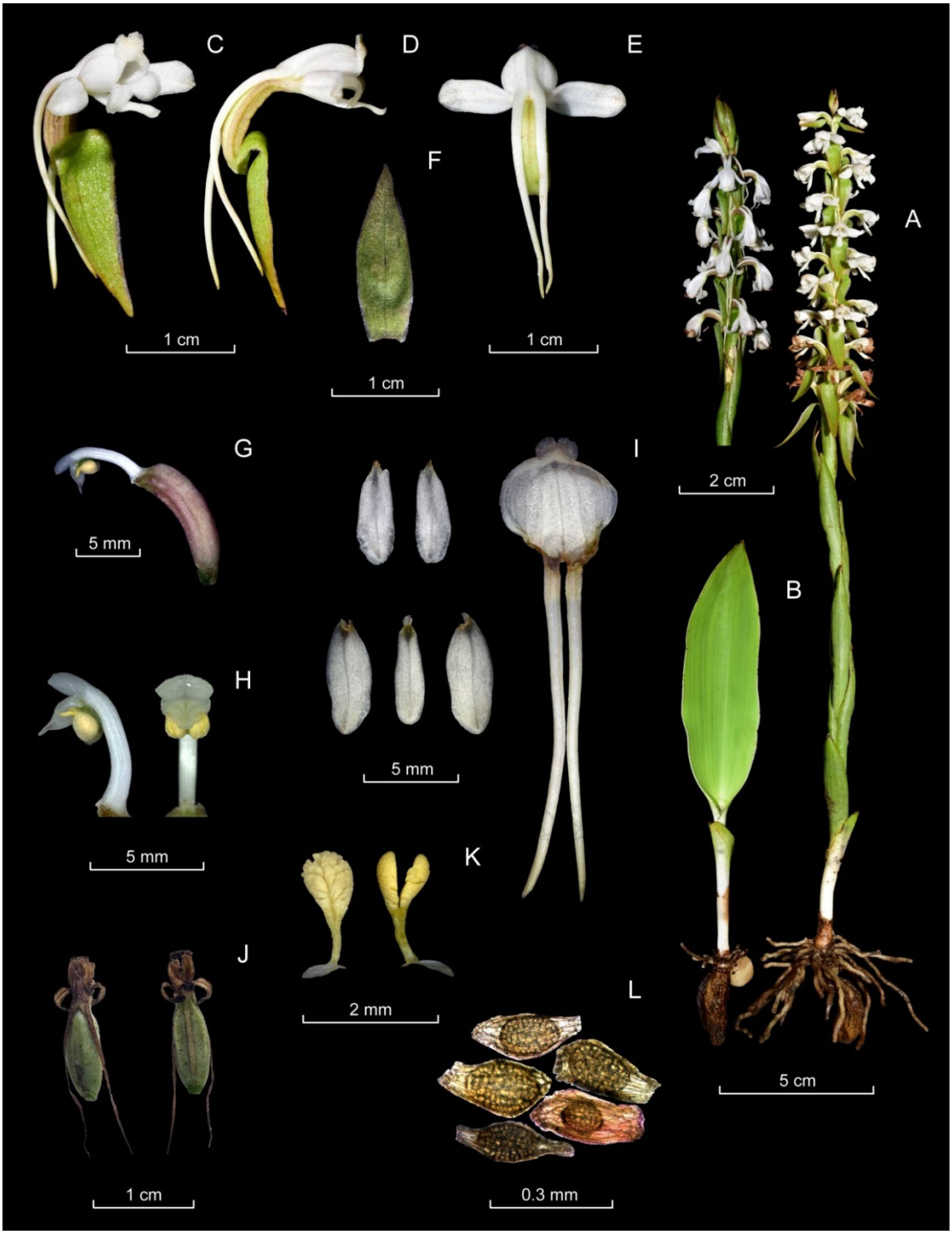

Figure 5.2. Lankester Composite Dissection Plate (LCDP) of *Satyrium longicauda* var. *redivivense* Castañeda-Zárate & van der Niet. A. Reproductive stem. B. Sterile stem. C. Flower oblique view. D. Flower side view. E. Flower dorsal view. F. Floral bract. G. Column and ovary in lateral view. H. Column in lateral and ventral view. I. Dissected perianth. J. Capsule in dorsal and ventral view. K. Pollinaria. L. Seeds. Based on photographs of specimens collected by M. Castañeda-Zárate 1671, 1675, 1731, and 1760.

Etymology: The specific epithet refers to *Rediviva* Friese (Melittidae), the dominant group of oil-collecting bees in southern Africa (Steiner and Whitehead, 1990). Unlike other taxa within the *Satyrium longicauda* complex that are pollinated by nocturnal moths that feed on nectar offered inside the long spurs, *Satyrium longicauda* var. *redivivense* is exclusively pollinated at day time by the oil-collecting bee *R. neliana* (Castañeda-Zárate et al., 2021; Chapter 2).

Taxon recognition: One of the most distinctive characters of *Satyrium longicauda* var. *redivivense* is the orientation of the flowers that face downwards as result of a slight curvature of the ovary. Other taxa that are part of the *S. longicauda* complex have flowers that project either more upwards or in a horizontal plane. Additionally, the combination of characters including sepals and petals that are not reflexed, short spurs devoid of nectar, and the presence of one basal erect elliptic to lanceolate leaf present on a sterile stem allows for identification of the variety. All other morphotypes grouped either within var. *longicauda* or var. *jacottetianum* that are found in the type locality offer nectar as reward in their spurs (Castañeda-Zárate et al., 2021; Chapter 2). *Satyrium longicauda* var. *redivivense* flowers in December and early January, only overlapping a couple of weeks with one of the other five morphotypes of the complex reported at the type locality (Castañeda-Zárate et al., 2021; Chapter 2).

Evolution: Based on phylogenetic analyses of nuclear and plastid DNA, accessions of *Satyrium longicauda* var. *redivivense* are nested inside a suite of accessions of other morphotypes of the *S. longicauda* species complex (Castañeda-Zárate et al., 2021; Chapter 2). The pollinator shift that is associated with the phylogenetic position of *Satyrium longicauda* var. *redivivense* is likely one of the main isolating mechanisms between the new variety and other co-flowering morphotypes of the *S. longicauda* species complex (Grant, 1994). The phylogenetic position is consistent with a scenario of ongoing progenitor-derivative speciation (Castañeda-Zárate et al., 2021; Crawford, 2010; Chapter 2).

Additional specimens examined. South Africa, KwaZulu-Natal: **2930 (Albert Falls)**:, uMgungundlovu district, Mpofana municipality: **Mt Gilboa Nature Reserve (-AD)**, in Drakensberg Foothill Moist Grassland, 6 Dec 2016, alt. 1610 m, *M. Castañeda-Zárate* MCZ-1412 (NU); 18 Dec 2016, alt. 1607 m, *M. Castañeda-Zárate* MCZ-1414 (NU, BOL, PRE); 13 Dec 2017, alt. 1607 m, *M. Castañeda-Zárate* MCZ-1488 (NU, BOL, PRE, NBG); 17 Dec 2017, alt. 1608 m, *M. Castañeda-Zárate*

MCZ-1494 (NU, BOL); 6 Dec 2018, alt. 1604 m, *M. Castañeda-Zárate* MCZ-1606 (NU, BOL); 19 Dec 2018, alt. 1603 m, *M. Castañeda-Zárate* MCZ-1614 (NU); 14 Dec 2019, alt. 1605 m, *M. Castañeda-Zárate* MCZ-1671 (NU); 9 Dec 2020, alt. 1609 m, *M. Castañeda-Zárate* 1731 (NU, BOL, PRE, NBG). **Karkloof Nature Reserve (-AD)**, in Drakensberg Foothill Moist Grassland, 20 Dec 2019, alt. 1624 m, *M. Castañeda-Zárate* MCZ-1675 (NU, BOL).

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CHAPTER 6

GENERAL DISCUSSION

Results presented in this thesis provide insight into various aspects of the systematics and evolution of *S. longicauda*. Variation in reproductive as well as in vegetative traits indicates that *Satyrium longicauda* comprises a species complex (Hall 1982). However, the current taxonomy of the species complex that recognises only two varieties (var. *jacottetianum* and var. *longicauda*), does not reflect the tremendous morphological variation observed in the group. Here, I have characterised the morphological and genetic variation, and pollination systems of natural populations found in South Africa. Eight morphotypes within *S. longicauda* that were defined *a priori* based on visual assessment of a combination of reproductive and vegetative traits, as well as *S. rhodanthum*, could be separated to various degrees in uni- and multivariate analyses, although substantial overlap in most evaluated traits was apparent. At sites where morphotypes co-occurred, these were frequently both morphologically and genetically distinct, suggesting they could be considered reproductively isolated units. At a broader geographical scale, the phylogenetic relationships among the morphotypes were partially resolved and only two morphotypes were recovered as monophyletic. Neither of these represent the morphotypes that served to describe the current taxa in *S. longicauda*.

The influence of pollinators in driving the morphological variation in the group was tested by describing the pollination systems of each morphotype and associating these with variation in various floral traits such as spur length, colour, scent, and nectar properties. As predicted by the pollination syndrome concept, most morphotypes received visits by nocturnal moths. However, pollination by other pollinator functional groups such as long-tongued flies, sunbirds, and oil-collecting bees was also documented. Surprisingly, populations of one of six morphotypes occurring in sympatry at one site differed from the other morphotypes in functional pollinator group and floral reward properties and type (nectar vs oil). Across the morphotypes, five modules representing pollination niches were identified by plant-pollinator network tools. Of these, the sunbird pollination module was the most divergent in traits, followed by the module characterised by oil-bee collecting pollination. The remaining three modules, characterised by various degrees of moth pollination, overlapped in most traits but subtle differences in functional spur length as well as nectar volume and concentration support the recognition of a hawkmoth module that differs from a settling moth module. Altogether, these results provide evidence for pollinator-driven diversification within a species complex.

Morphological and genetic variation and their taxonomic implications

Plant species with a broad geographical distribution often exhibit a variable phenotype as a result of changes in environmental conditions, local pollinator assemblages, mating systems, and other selective agents acting on natural populations (Herrera *et al.* 2006; Nattero and Cocucci 2007; Hodgins and Barrett 2008; Nattero *et al.* 2011; Arista *et al.* 2013; Schouppe *et al.* 2017; Guerra *et al.* 2019). A first strategy to identify the processes, mechanisms, and agents involved in shaping the phenotypic and likely genetic differences in natural populations, requires the characterisation of natural variation at different spatial scales through the implementation of a series of tools and methods. The selection of methods will depend on the system and the type of variation that is being evaluated (Schlick-Steiner *et al.* 2010). However, a first and basic approach to quantify morphological variation detected by initial visual evaluation requires the characterisation of phenotypes by means of traditional morphometrics and uni- and multivariate analyses, or the implementation of geometric morphometrics tools describing the patterns and magnitude of the morphological variation (Mutanen and Pretorius 2007). In lineages which are characterised by variation in specialised pollination systems, such as orchids in general, floral characters are often divergent and play an important role in systematic treatments, and are therefore commonly characterised (Anderson *et al.* 2002). The information gathered through morphometric techniques, such as the identification of morphological discontinuities, is not only valuable in taxonomy and systematics, but could also be useful in fields such as ecology and evolution, as it provides a starting point for investigating potential drivers of such variation. Subsequent analyses can then be implemented to evaluate particular hypotheses.

The large phenotypic variation observed in *S. longicauda* that had not hitherto been evaluated, indicates the likely existence of more than two varieties or even species as part of the complex. Multivariate analyses demonstrated that the large variation in vegetative and reproductive traits allowed the recognition of different morphotypes. In spite of their floral and vegetative differences detected by visual evaluation of qualitative differences, the morphotypes overlapped in several of the quantified traits. Although these morphotypes show no significant morphological discontinuities that can be implemented in the current taxonomy of the group proposed by Hall (1982), they do provide a more complete and clear picture of the overall variation observed in the group. Previous researchers recognised var. *longicauda*, var. *jacottetianum*, and *S. buchananii* (van

der Niet *et al.* 2005; van der Niet and Linder 2008). The phylogeny reconstructed here, based on the nuclear ITS sequences of the morphotypes within the complex, revealed that the OELB and TELW morphotypes form monophyletic clades, but other morphotypes and *S. longicauda* as a whole are not monophyletic. The recognition of morphotypes that were previously unknown within the two varieties recognised by Hall (1982), and the addition of *S. rhodanthum* to the species complex represent a first attempt to establish the phylogenetic relationships within the complex (Chapter 3) and provides a framework that can form the foundation for further taxonomic studies.

Extensive variation in the foliage leaves of the vegetative and reproductive stem had served to differentiate between groups of *Satyrium* (Hall 1982; Kurzweil and Linder 1999), but most systematic studies in orchids focus on floral traits. However, the morphotypes within the complex here identified can be delimited by a combination of floral traits such as spur length and other quantitative traits, together with quantitative and qualitative vegetative traits such as the number and position of leaves, and size. Although variation in vegetative traits might represent a plastic response to environmental conditions (Franks *et al.* 2014; Moran *et al.* 2016; Villellas *et al.* 2021), and has therefore been neglected in systematic studies, the correct identification of morphotypes within *S. longicauda* depends on the number and position of leaves on the sterile shoot (Hall 1982). Interestingly, preliminary results of *in vitro* seed germination trials showed that seedlings have leaves that are similar in shape, number and orientation to adult plants (M. Castañeda-Zárate, unpubl. data), suggesting that variation in leaf number has a genetic basis, as also indicated by the maintenance of these morphotype differences in sympatric populations (although the influence of microhabitat on phenotypic plasticity cannot entirely be excluded in field-based studies of sympatric forms). Sampling of multiple individuals in natural populations represented a key strategy implemented here for the characterisation of intraspecific variation of *S. longicauda*. This approach provides more information than what it is typically present on a herbarium specimen; for instance, because collectors usually only collect the inflorescence and not the leaves.

Pollinators as agents of morphological variation in the flowers of *Satyrium longicauda*

Several studies have provided evidence that variation in floral morphology in *Satyrium* is the result of adaptations to different pollinators (Johnson 1997; Johnson *et al.* 2011). The extensive list of floral visitors and pollinators of *S. longicauda* reported in this thesis revealed that moth-pollination, which was previously reported in several studies (Johnson *et al.* 2005, 2009, 2011; Jersáková and Johnson 2007; Ellis and Johnson 2010; Duffy and Johnson 2014), is predominant among the morphotypes here identified. However, the broad sampling implemented here aided in the discovery of pollination systems known for other *Satyrium* species, but not for *S. longicauda*, viz. pollination by long-tongued flies, sunbirds and oil-collecting bees. These pollination systems deviate from expectations based on the pollination syndrome concept (Faegri and van der Pijl 1979; Vogel 2012), and may indicate that the populations of OELM that utilise pollinators belonging to two different pollinator groups (moths and long-tongued flies or moths and sunbirds) may be undergoing an ongoing evolutionary shift involving an "intermediate stage of double function" (Stebbins 1970) that may eventually give rise to more discrete pollination ecotypes (Table 1.1).

Pollinator shifts, i.e. the adaptation of plant populations to a new pollinator, are considered common and widespread, therefore they are important for angiosperm diversification (van der Niet and Johnson 2012). Floral traits involved in the evolution of pollination ecotypes other than those regarding mechanical fit between pollinators and flowers, include variation in scent and nectar as reward (Table 1.1). However, pollinator shifts driven by the change from one type of reward to another are rare (but see Armbruster and Baldwin 1998). Here, I have shown that although the OELB morphotype has completed the transition from moth to oil-collecting bee pollination, thereby representing a different pollination ecotype, it retains traits related to moth-pollination (Chapter 1). Similar to almost all other morphotypes included here, the OELB morphotype exhibits floral traits characteristic of moth-pollination. However, a key difference between this morphotype and the predominantly moth-pollinated morphotypes is the larger amount of diacetin produced by its flowers and the almost complete lack of a nectar reward. Diacetin, an oil-derived compound that is considered to function as a private communication channel between oil-collecting bees and oil-producing flowers (Schäffler *et al.* 2015), was present

only in trace amounts in moth-pollinated morphotypes and likely represents a pre-adaptation for pollination by oil-collecting bees.

Intraspecific variation found among the *S. longicauda* morphotypes is indicative of differences in pollination system. If pollination systems are mapped onto the phylogeny presented here, moth-pollination appears to represent the ancestral pollination system of the complex whereas oil-collecting bee pollination represents a derived state (Chapter 2; Chapter 3). Based on the presence of diacetin in all morphotypes for which this was measured, a shift in pollination system from pollination by moths to oil-collecting bees may be due to an increase in the production of oils and their volatile by-product diacetin. Additionally, the embedded position of *S. rhodanthum* in the phylogeny within *S. longicauda* clades, is consistent with a shift from pollination by moths to sunbirds, potentially driven by changes in flower colour and cessation of scent emission. Interestingly, this shift is in the opposite direction of the shifts from pollination by hummingbirds to moths in the North American genus *Aquilegia* (Whittall and Hodges 2007). An explanation for this shift may be that sunbirds opportunistically feed on plant species adapted for different specialised pollination systems (Rebelo 1987; M. Castañeda-Zárate, pers. obs.), which may drive selection for bird pollination if the original pollinator is temporarily absent.

Coexistence

Since species do not occur as isolated entities but are part of a community (Chesson 2000), competition and facilitation stand as the processes that affect their coexistence, and these processes may have important evolutionary consequences (Palmer *et al.* 2003; Bürger *et al.* 2006; Hochkirch *et al.* 2007; Sargent and Ackerly 2008). Thus, identifying the mechanisms that allow taxa to coexist is fundamental, not only in evolutionary biology but also has implications for taxonomy and conservation (Chesson 2000; Ennos *et al.* 2005; Bickford *et al.* 2007; Hart *et al.* 2017). For instance, multiple coexisting forms of a species complex could be considered biological species, and may therefore be elevated as taxonomic species. Similarly, recognition of multiple coexisting species means that conservation priorities may need to be reconsidered. In plants, the mechanisms that allow coexistence and promote diversification of populations include polyploidy, mutualisms with pollinators, mycorrhyzal symbiosis, differences in phenology, preference for particular habitats, and biogeographic history (Stevens 1989; Chesson 2000; Ishii and Higashi 2001;

Sargent and Ackerly 2008; Waterman *et al.* 2011; Pauw 2013; Germain *et al.* 2016). Ecological theory predicts that species must occupy specific ecological niches to reduce competition to a level where stable coexistence is possible (Silvertown 2004; Pauw 2013; Phillips *et al.* 2020).

Populations of different morphotypes of *S. longicauda* are commonly found in sympatry and they sometimes show similarity (and sometimes differences) in pollinator assemblages; however, subtle differences in flowering phenology may prevent natural hybridization from occurring. Interspecific experimental hand pollinations between different morphotypes revealed absence of postzygotic isolation, based on high fruit set, seed viability, and seed germination by *in vitro* techniques (M. Castañeda-Zárate, unpubl. data). Nevertheless, morphotypes that produce hybrids may be postzygotically isolated through hybrid sterility resulting from polyploidy. However, preliminary results from flow cytometry analyses on several morphotypes revealed that only one morphotype is polyploid (van der Niet, T, unpubl. data). Therefore, morphotype integrity likely relies on prezygotic barriers. Differences between spur length and proboscis length may represent a mechanical isolating mechanism among forms, since pollinarium attachment on different sections of the proboscis of the pollinator could prevent interspecific pollen transfer (cf. Grant 1994; Waterman *et al.* 2011). The mechanism that allows the OELB morphotype to coexist with other members of the complex, may include the utilisation of a different pollinator through which this morphotype is reproductively isolated from other sympatric morphotypes through behavioural and temporal isolation. Additionally, preliminary phenology observations indicate that differences in flowering time exist among sympatric morphotypes. For instance, at the Mt Gilboa site, an earlier flowering time of the OELB morphotype compared to primarily moth-pollinated morphotypes, which among one another also have differences in flowering phenology, may also represent a prezygotic barrier that prevents morphotypes from interbreeding (M. Castañeda-Zárate, unpubl. data). The field-based evidence strongly suggests that the OELB morphotype is reproductively isolated from other morphotypes, and this is further supported by its monophyly and unique ITS haplotypes, suggesting that it is a valid species based on the Biological Species Concept (Mayr 1942, 1963). However, the mostly continuous nature of the morphological variation that is used to diagnose this morphotype rather supports the description of a new variety that is distinct from varieties *jacottetianum* and *longicauda* (Chapter 3) and not a new species, based on the Typological Species Concept. This tension in taxon definition based on differences between species concepts can be resolved by further research into the genetics of this

morphotype and other morphotypes, as well as a more complete revision of the species complex, and may ultimately elevate this variety to the species level. At a community scale, the identification of discrete pollination niches may explain how different morphotypes of *S. longicauda* that occur in sympatry, and have partial overlap in flowering time, can coexist (Castañeda-Zárate *et al.* 2021; Chapter 2). The use of pollinators with contrasting foraging strategies, combined with differences in phenology, represent potentially important reproductive isolation barriers, facilitating the diversification of the group.

Further avenues for future research

Further studies could be performed to arrive at stronger conclusions about the role of pollinators in plant speciation and may have implications for species delimitation (Sites and Marshall 2004; Coates *et al.* 2018). These studies could focus on the characterisation of functional floral traits of sympatric and allopatric populations, and could involve a more extensive sampling of populations and individuals for analyses that combine genomics and detailed population genetic studies using various types of molecular markers to determine genetic diversity within and among morphotypes (Duminil and Di Michele 2009; Hausdorf and Henning 2010; Idrees and Irshad 2014). Moreover, studies that track pollen fates among co-flowering sympatric morphotypes using histochemical dyes (Peakall 1989; Johnson *et al.* 2005) may be used to quantify the degree of pollinator isolation, by assessing whether pollinator only move pollen within morphotypes or whether morphotypes that share the same pollinator attach pollen on different parts of pollinators, thereby allowing coexistence. These analyses could be supplemented with paternity analyses to characterise realised hybridization rates (Bernasconi 2003; Figueroa-Castro and Holtsford 2009; Rymer *et al.* 2010; Pollegioni *et al.* 2013). Other potential mechanisms that are worth studying include the characterization of mycorrhizal fungus communities, as these may differ among morphotypes, thereby reducing competition for resources (van der Heijden *et al.* 2003; Waterman *et al.* 2011; Jacquemyn *et al.* 2014; McHaffie and Maherali 2020). Additionally, the study of microhabitat segregation (specific ecological niche) may be used to assess the importance of habitat specialization for reducing competition that facilitates species coexistence (Tubay *et al.* 2015; Paudel *et al.* 2018; Mantel and Sweigart 2019).

Conclusion

Results presented in the present thesis are based on a combination of studying reproductive biology and plant systematics, and may result in "reciprocal illumination" (Anderson *et al.* 2002). This process as a means of integrative taxonomy (Marcussen and Borgen 2011; Vislobokov *et al.* 2013; Hu *et al.* 2018; Bastiani *et al.* 2020; Botes *et al.* 2020), may provide more insight into the taxonomy of the group. New information from different disciplines will lead to a more comprehensive taxonomic revision of the complex by identifying characters that could potentially be used to diagnose taxa. This may result in an updated nomenclature, and delimitation and description of recognised and unrecognised taxa that can be implemented in conservation programs. At the same time, an updated taxonomy may provide a starting point for studies of the evolution of these units. This work could also serve as a reference for the study and understanding of other species complexes, not only within the genus *Satyrium* but other plant groups that exhibit substantial morphological variation across their geographical distribution and are currently undergoing speciation.

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