Pollination, floral deception and evolutionary processes in *Eulophia* (Orchidaceae) and its allies

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Thesis

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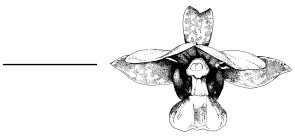

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For E

a. Abstract

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Orchids provide a model system for addressing evolutionary and ecological questions both because of their species diversity, and because the packaging of their pollen into pollinia facilitates the estimation of male and female pollination success. This thesis focuses on the ecology and evolution of pollination systems in the African orchid genus *Eulophia*, with an emphasis on deceptive pollination, mechanisms promoting cross-pollination, and pollinator-driven speciation.

Pollination in the deceptive species *E. zeyheriana* is shown to depend on flower colour and proximity to the rewarding model species, *Wahlenbergia cuspidata* (Campanulacae). This study demonstrates the functional importance of colour matching between model and mimic in a floral Batesian mimicry system, as well as the importance of facilitation by the rewarding model [chapter 2].

The pollinaria of the vast majority of *Eulophia* and *Acrolophia* species undergo reconfiguration following removal by pollinators, similar to the phenomena first described by Darwin in some European orchids and which he hypothesised to be adaptations to limit pollinator mediated self-pollination. In chapter 3, a less common mechanism – anther cap retention – is described for *E. foliosa*. Observations of reconfiguration times were compared to the respective visit times by pollinators in a number of orchids (including *Eulophia* and *Acrolophia*) and asclepiads. In 18 of 19 species, pollinarium reconfiguration times exceed the average visit times, providing empirical support for Darwin's cross-pollination hypothesis [chapter 4].

All of the 25 species of *Eulophia* examined are deceptive, but two of the three species in the small, closely related Cape genus *Acrolophia* examined in chapter 5 are rewarding. This translates into very high levels of pollen transfer efficiency in the rewarding *A. cochlearis* relative to the deceptive *A. capensis* and species of *Eulophia*. In addition, *A. cochlearis* exhibits high rates of pollinator-mediated self-pollination, as quantified using a novel method based on levels of inbreeding depression during embryo development.

In chapter 6 the evolutionary divergence of long- and short-spurred forms of *E. parviflora* in response to different pollinators is investigated. This shows that divergence has occurred in floral morphology, scent chemistry and flowering phenology and that this can be attributed to adaptations to the respective bee and beetle pollinators of each form.

This thesis also includes case histories of bee pollination in an additional five *Eulophia* species, and beetle-pollination in two other species of *Eulophia* with dense inflorescences and slow pollinarium reconfiguration [chapter 7]. In addition, four taxa were found to undergo auto-pollination [chapter 8].

The main conclusions of this thesis are that pollination of food-deceptive species can be enhanced by spatial proximity to, and floral colour matching with, sympatric rewarding species; that selection strongly favours traits that promote cross-pollination; that pollinator-shifts can drive speciation; and that floral adaptations for bee-, beetle-, and auto-pollination are found in South African representatives of *Eulophia*.

b. Declaration

I, Craig Ingram Peter declare that this thesis is my own work and has not been submitted in part or in whole to any other University. Where use has been made of the works of others, this has been acknowledged in the text through citation of the original source.

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c. Acknowledgements

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- And finally to Kerry and Ethan. This thesis was only possible because of you.

d. Contents

Appendix 1– Related papers

[chapter 1]

Introduction

DECEPTIVE POLLINATION

Deceptive pollination systems are known from at least 32 angiosperms families (Renner 2006). However the vast majority of deceptive species belong to the Orchidaceae (van der Pijl & Dodson 1966, Ackerman 1986). Van der Pijl & Dodson (1966) estimated that one third of all orchids (roughly 8000 species by their estimate) were deceptive. Ackerman (1986) used the estimate of van der Pijl & Dodson (1966) to increase the number of deceptive species to 10 000 species, presumably basing his estimate on a revised figure for the number of species in the family.[1]

The extensive reviews of pollination in the Orchidaceae by van der Cingel (van der Cingel 1995, van der Cingel 2001) allow us for the first time to estimate the percentage of orchids that are deceptive based on actual pollination studies. In addition to scoring the pollinators of different species recorded in these two works, subsequent peer-reviewed literature and the current research, as described later in this Introduction (pg 15), I scored species which are recorded as having a reward or not. Species where the presence or absence of a reward is not mentioned were not included in the data set. Auto-pollinating and agamospermous species were excluded from this analysis as the presence of nectar may be inherited from allogamous ancestors, while the absence of nectar may be a result of selection in the absence of animal pollinators. Reward is interpreted broadly as including nectar, pseudopollen and glandular hairs, oils, waxes, resins and fragrant compounds. Of the 734 species for which data on rewards are available, 58.4% are rewarding opposed to 41.6% that are deceptive, comparable with, but higher than, the estimates of van der Pijl & Dodson (1966).

[1] Dressler (1981) is widely cited as a source for the estimation that a third of all orchid species are deceptive. However, I did not find any reference to this statistic in his book. A later publication (Dressler 1993) does not cite the earlier book, relying on the estimate of van der Pijl & Dodson (1966). Gill (1989) also relies on this estimate of van der Pijl & Dodson, and notes that all of the subfamilies have non-rewarding species, while the Cypripedioideae are exclusively deceptive.

Different types of deception

Deceptive systems in orchids include Batesian mimicry of rewarding plant species, generalised food deception systems in which plants exploit the foraging of naïve pollinators and sexual deception systems in which flowers mimic the cues that female Hymenoptera use to attract male insects. These mechanisms have been recently reviewed by Schiestl (2005) and Jersáková *et al.* (2006). In addition, Jersáková *et al.* (2006) review less common mechanism of deception such as brood-site mimicry, shelter imitation, pseudoantagonism and rendezvous attraction.

The adaptive significance of deception

Charles Darwin did not accept the phenomenon of rewardlessness in orchids as he believed that insects would rapidly learn to avoid such flowers and because he was convinced that nectar was contained within the tissue of the apparently empty floral spurs of many European orchids (Darwin 1867). However, subsequent research has shown that Sprengel (1750 – 1816; cited in van der Cingel 1995) was right to conclude that many orchids are deceptive. A number of hypotheses have been proposed to explain deception in orchids although only three of these have received much attention.

The resource-limitation hypothesis explains deception in terms of the physiological savings for plants not having to produce nectar when resources are limiting (Ackerman 1986). However, many deceptive orchids are pollen limited (Tremblay *et al.* 2005) and there is evidence "that a meagre nectar offering … is better than nothing" in attracting pollinators (Ackerman *et al.* 1994, p 44), thereby reducing pollen limitation at minimal cost. In addition, many deceptive orchids produce copious amounts of extra-floral nectar, suggesting that the cost of nectar *per se* cannot explain the phenomenon of empty flowers.

The pollen export hypothesis proposed by Smithson and her co-workers (e.g. Smithson & Gigord 2001) proposes that rewardlessness enhances pollinaria removal. Although supported by an experiment in which removal of pollinaria of *Barleria robertiana* decreased with nectar supplementation (Smithson & Gigord 2001, Smithson 2002), most subsequent nectar supplementation studies have found an association between nectar and removal of pollinaria. In addition, broad surveys of orchids show an association between nectar rewards and high levels of pollinarium removal (Harder 2000).

The cross-pollination hypothesis proposes that deception in orchids serves to reduce geitonogamous pollen transfer, thereby maximizing cross-pollination even though overall levels of fruit set may be pollen limited. Pollinators that do not find a reward spend less time and probe fewer flowers on non-rewarding inflorescences, promoting cross-pollination between plants (Dafni & Ivri 1979, Dressler 1981, Jersáková *et al.* 2006). In comparison to the uncertainty surrounding the resource limitation and pollinarium export hypotheses, the cross-pollination hypothesis has received empirical support from a number of nectar supplementation studies (Johnson & Nilsson 1999, Johnson *et al.* 2004 [appendix 1b], Jersáková & Johnson 2006).

Facilitated pollination

Interactions between individual plants have typically been viewed within the paradigms of intra- and inter-specific competition and several studies have shown that plants can compete for pollinators (Levin & Anderson 1970, Schemske *et al.* 1978, Waser 1978a, Waser 1978b, Caruso 1999).

There is, however, increasing recognition that facilitation is an important process in many ecological interactions, including those involved with pollination (Feinsinger 1987, Callaway 1995, Feldman *et al.* 2004). Batesian floral mimicry systems are obvious examples where rewarding model species facilitate the pollination of deceptive mimicking species (cf. Nilsson 1983, Johnson 1994, Johnson 2000, Anderson *et al.* 2005, Peter & Johnson 2008 [chapter 2]). In addition, Thomson (1978) proposed the so-called magnet species effect where a rewarding species may increase the local pollinator abundance in an area. Reproductive success of co-occurring species (including two or more rewarding species, rare species and species with inferior rewards or deceptive species) may be enhanced due to the increased local pollinator densities attracted to the one or more "magnet species".

Moeller (2004) explains this form of facilitation through a "joint attraction of pollinators mechanism" which is compatible both with the magnet effect benefiting species with inferior rewards and mutual attraction of two or more rewarding species both benefiting by attracting mobile pollinators into an area. A second process, the "joint maintenance of pollinators" hypothesis, explains how coexisting species jointly provide resources to resident pollinators (Moeller 2004). This may take the form, for example, of staggered flowering of

different rewarding species through a season, maintaining larger more consistent rewards to the local, specialised pollinators (Moeller 2004, Moeller 2005).

In contrast to these examples where the interacting species show a degree of similarity, Ghazoul (2006) provides evidence that some pollinators may be attracted to diverse floral assemblages. This is explained in terms of optimal access to multiple rewards from the different facilitating species.

A number of studies have demonstrated that magnet species can have a positive effect on the reproductive success of co-occurring species. These studies examine primarily the influence of magnet species on the reproductive success of co-occurring rewardless species. Laverty (1992), for example, showed that rewardless *Podophyllum peltatum* had significantly higher seeds set when they co-occurred with rewarding *Pedicularis canadensis* plants compared to *P. peltatum* plants growing alone. In contrast, Alexandersson & Ågren (1996) found that pollen export from the deceptive orchid *Calypso bulbosa* was positively related to the co-occurring rewarding species, *Salix caprea*, in only one of the three years of their study.

More recently, Johnson *et al.* (Johnson *et al.* 2003b [appendix 1a]) demonstrated that the deceptive European orchid *Anacamptis morio* has significantly higher rates of pollen removal and deposition when transplanted into patches of two very different nectar-producing magnet plants (*Geum rivale* and *Allium schoenoprasum*). At larger spatial scales, they demonstrate a significant positive relationship between the density of these two magnet species and visitation to the transplanted orchids in $100m^2$ plots as well as overall nectar plant densities at the scale of whole meadows.

Johnson *et al.* (2003b [appendix 1a]) also show that bumble bee pollinators are more likely to investigate deceptive orchid inflorescences if they have just fed on a rewarding species with flowers that are similar in colour to those of the orchid. Similarly, Gumbert & Kunze (2001) show that bees are more likely to shift from flowers of a rewarding species to those of the deceptive orchid *Anacamptis (Orchis) boryi* if they have similar flower colour than if they don't. These observations correspond with more general observation that pollinators are more likely to switch between similar coloured rewarding species than between rewarding species of different colours (Chittka *et al.* 1997).

Based on these observations, Johnson *et al.* (2003b [appendix 1a]) proposed a continuum between non-rewarding species that incidentally resemble co-occurring nectar plants and benefit from these rewarding plants as a result of the magnet effect; and the examples of floral Batesian mimicry where mimics are often remarkably similar to their rewarding models in colour, shape and perhaps scent. In such a pathway, natural selection would increasingly favour phenotypes of the deceptive species that superficially resemble a co-occurring rewarding species, driving the advergent evolution of the deceptive species to ultimately resemble the rewarding species both in terms of morphology, colour and perhaps scent.

Importance of colour and fragrance in deceptive systems

The majority of studies on floral Batesian mimicry systems have focused on documenting the similarity of the colour of the mimic to that of the model (e.g. Nilsson 1983, Johnson 1994, Johnson 2000, Galizia *et al.* 2005, Anderson *et al.* 2005). However, these studies have been based purely on descriptions of floral spectral reflectance patterns. Peter & Johnson (2008 [chapter 2]) demonstrated experimentally for the first time that altering the colour of the mimic's flowers disrupts its exploitation of the rewarding model.

Fewer studies have explicitly considered the importance of inflorescence size (Galizia *et al.* 2005) and shape (Johnson *et al.* 2003a) or scent (Galizia *et al.* 2005) in floral Batesian mimicry. Scent mimicry in flowers appears to be limited to cases of sexual and brood site mimicry (Dettner & Liepert 1994).

MECHANISMS THAT PROMOTE CROSS-POLLINATION IN ORCHIDS

Most orchids (and "asclepiads") package their pollen into pollinia and as a result are susceptible to self-pollination which has three potentially serious consequences. Firstly, self-deposition of an entire pollinium may eliminate most or all of the opportunity for a flower's pollen to be exported. This process is known as pollen discounting (Barrett 2002). Secondly, for self-compatible species, such as most orchids, ovules self-fertilized en masse are rendered unavailable for cross-fertilization, a process known as ovule discounting (Herlihy & Eckert 2002). Thirdly, self-fertilization typically results in significant inbreeding depression (Darwin 1876, Charlesworth & Charlesworth 1987) and in the orchids, many species show rates of embryo abortion in selfed seeds that are double those in seeds arising from cross-fertilization (Tremblay *et al.* 2005).

Many orchids and asclepiads have mechanisms that are thought to protect against these unfavourable outcomes of self-pollination. Pollinarium reconfiguration is perhaps the best recognised of these mechanisms. Since Darwin's (1867) observations of bending reconfigurations in a variety of European and tropical orchids, many authors have described bending reconfigurations in numerous different orchids (e.g. Kullenberg 1961, van der Pijl & Dodson 1966, Cole & Firmage 1984, Johnson & Nilsson 1999, Ayasse *et al.* 2000). Additional modes of pollinarium reconfiguration have been described including pollinium shrinkage (Borba & Semir 1999), anther cap retention (Catling & Catling 1991) and pollinarium bending in some "asclepiads" (Queller 1985). Aditional mechanisms that limit self-pollination in orchids include the rare examples of dioecy (Romero & Nelson 1986) and stigma reconfigurations, as in the case of *Listera ovata* (Darwin 1867).

Darwin was the first to hypothesise that such pollinarium reconfigurations may serve to protect against self-pollination (Darwin 1867) with freshly removed pollinaria being incorrectly orientated to make contact with the stigma. Following a period that Darwin suggested would exceed the visit time of the pollinator, the pollinarium bends or is "depressed" into a new position where the pollinia may be deposited. While many studies have described pollinarium reconfigurations only a handful have attempted to compare reconfiguration times with pollinator visit times to test Darwin's hypothesis (Cole & Firmage 1984, Catling & Catling 1991, Borba & Semir 1999, Johnson & Nilsson 1999, Ayasse *et al.* 2000). To date, only Johnson *et al.* (2004 [appendix 1b]) have been able to show experimentally that self-pollination does not occur unless the visit time of the pollinator exceeds the bending time of the pollinarium in a single European species.

POLLINATOR-DRIVEN DIVERGENCE

Pollinators are widely recognised to be one of the driving forces of the evolution of the massive floral diversity in the Angiosperms (Dodd *et al.* 1999, Kay *et al.* 2006) through the adaptation of flowers to the large diversity of flower-visiting animals that pollinate them.

Johnson (2006) has recently synthesised the ideas of Grant & Grant (1965) and Stebbins (1970) into what he calls the "Grant-Stebbins model" for pollinator-driven speciation. In this model a patchy pollinator fauna, typically reflecting a geographic pattern (Herrera *et al.* 2006), may result in different populations of a plants species becoming adapted to

different pollinators through the most efficient pollinator principle (Stebbins 1970), resulting in two or more pollination ecotypes. Ultimately such divergent specialisation for different pollinators in different populations may lead to reproductive isolation and speciation when the differences between ecotypes become sufficiently distinct to prevent subsequent hybridization (Johnson 2006).

Pollination ecotypes

Despite the simplicity of this model for pollinator driven speciation, empirical evidence to support it is limited to microevolutionary studies of selection imposed by pollinators (Irwin & Strauss 2005, Morgan 2006, Conner 2006) and studies showing macroevolutionary radiations of lineages in response to different pollinators (Givnish & Sytsma 1997, Johnson *et al.* 1998, Soltis *et al.* 2005). Studies showing the intermediate stage – the evolution of pollinator ecotypes – are rare and limited to studies showing the presence of correlations between the floral traits of ecotypes and the morphology and behaviour of their respective pollinators (Johnson 2006, Herrera *et al.* 2006).

Studies showing such "trait-environment" correlations primarily focus on adaptation of floral tubes and spurs to pollinators with variable proboscis lengths (Robertson & Wyatt 1990, Johnson 1997, Johnson & Steiner 1997, Boyd 2004). Less common traits showing such divergences between ecotypes include flowering phenology (Herrera *et al.* 2002), scent chemistry (Galen 1985, Pellmyr 1986, Johnson *et al.* 2005) and colour (Johnson 1994). Experimental evidence for pollinator-ecotypes has been limited to a handful of studies of pollinator foraging preferences (Galen 1989, Robertson & Wyatt 1990) and effects the of trait modification (Johnson & Steiner 1997).

Diversity of orchids and their pollination systems

The family Orchidaceae is one of the largest and most morphologically diverse families of Angiosperms with estimates of the number of species ranging from about 19 000 (Atwood 1986, Dressler 1993) to 25 000 species (Chase 2005). Much of this diversity can be attributed to the fact that the flowers are adapted to specialist pollination by a wide range of animal pollinators (van der Pijl & Dodson 1966, Dressler 1981). Orchids provide a model system for evolutionary and ecological studies because of this exceptional diversity (accumulated since the late cretaceous, 76-84 Myr ago; Ramirez *et al.* 2007) and the fact that pollen is packaged as pollinia, allowing researchers the opportunity to gauge pollination success much more easily than is the case with most other plants. Interest in the pollination

biology of orchids goes back to Sprengel (1750 – 1816), the father of flower biology who was the first to recognise that orchids may deceive their pollinators by not offering them a nectar reward (van der Cingel 1995). However, the most famous champion of orchid pollination research was Darwin who devoted an entire book to the subject (Darwin 1867) which still provides inspiration to current-day researchers. In the twentieth and twenty-first centuries, particularly since the 1960's, a huge research effort has been devoted to aspects of the pollination biology of this family. However, the last major synthesis of orchid pollination research was by van der Pijl & Dodson (1966). Van der Cingel (1995, 2001) catalogued almost all published interactions between orchids and their pollinators, but did not provide a synthesis.

To estimate the importance of different groups of pollinating animals in the Orchidaceae, I included all species from these two works (van der Cingel 1995, van der Cingel 2001) where it is clear that at least one pollinator bearing pollinaria has been collected or observed. Definitions of groups of pollinators conform to the broadly accepted pollination syndromes (van der Pijl 1961, Fenster *et al.* 2004) with a few additional groups included. These are given in Table 1. Where one or more species of the same group of pollinators were recorded, the orchid was scored as being pollinated by that insect order or group. Where more than one of these pollination groups was recorded as pollinators, that species was scored as being a generalist. This is a broad definition of generalist pollination systems (cf. Johnson & Steiner 2000).

In addition, studies published since 1994 in peer-reviewed literature and included in either the Scopus or ISI web of science databases were added to this dataset where they are not included in the works of van der Cingel (1995, 2001).

This analysis indicates that pollinators have been identified for 1280 species from around the globe representing only about 5% of the approximately 25 000 species of orchids (Chase 2005). Sexual deception in the European genus *Ophyrs* (73 taxa) as well as the various scent rewarding Catasetinae, Maxillariinae and Oncidiinae (183 taxa) pollinated by Euglossine bees in South America are among the best studies orchid pollination systems.

The analysis shows that of the orchids with known pollination systems, approximately 40% are bee-pollinated (Table 1). The second most frequent category is auto-pollination at 30% of species. However, auto-pollination is almost certainly over-represented because auto-

pollinating species are more readily recognised and observations are required only on the flowers and not the interaction with the pollinators. In addition, and as described in chapter 8, caution needs to be exercised in the absence of observed mechanism when describing species with high levels of fruit set as auto-pollinating because insect parasitization may stimulate the development of capsules in unpollinated flowers. van der Pijl & Dodson (1966) estimated that just 3% of orchids undergo auto-pollination, while Catling (1990) estimated the figure to be between 5 and 20%. Given these uncertainties in estimating the actual occurrence of autogamy in orchids, the analysis was repeated for xenogamous taxa (i.e. excluding auto-pollinating species). In this case, bees account for the pollination of nearly 60 % of taxa (Table 1). Pollinators of secondary importance include wasps, flies, birds and both settling and sphingid moths. Nearly 5% of the examined taxa are broad generalists being pollinated by two or more of these groups of pollinators.

Table 1: Known pollinator frequencies in the Orchidaceae.

	All species		Xenogamous species	
	n	%	n	%
Auto-pollination	395	30.9		
Bees	515	40.2	515	58.2
Wasps	88	6.9	88	9.9
Flies	61	4.8	61	6.9
"Sapromyophily"	1	0.1	1	0.1
Long-tongued flies	12	0.9	12	1.4
Mosquitoes	4	0.3	4	0.5
Fungus gnats	18	1.4	18	2.0
Birds	49	3.8	49	5.5
Settling Moths	32	2.5	32	3.6
Sphingid Moths	27	2.1	27	3.1
Butterflies	18	1.4	18	2.0
Beetles	13	1.0	13	1.5
Ants	1	0.1	1	0.1
Thrips	1	0.1	1	0.1
Aphids	3	0.2	3	0.3
"Generalist"	42	3.3	42	4.7
total	***1280***		***885***	

THE STUDY SYSTEM AND HYPOTHESES

This thesis explores the ecology and evolution of pollination systems, with an emphasis on deceptive pollination, mechanisms promoting cross-pollination and pollinator-driven speciation, using the orchid genus *Eulophia* as a model system.

Eulophia is a large genus of approximately 230 terrestrial species (Thomas 1998), the majority of which are found in central and southern Africa. Although primarily African, there are also a number of species in India and south-east Asia with a single species common to central America and west Africa (Hall 1965). Linder & Kurzweil (1999) consider the genus *Eulophia* as possibly the most important orchid genus in the African savanna. Species of this genus are found in all major terrestrial habitats in South Africa.

The taxonomy of the genus and its taxonomic affinities is currently confused (Thomas 1998). Dressler (1981) included *Eulophia* and the small Cape genus *Acrolophia* along with genera such as *Ansellia*, *Cymbidium*, *Oeceoclades*, *Pteroglossaspsis* and *Gramatophyllum* in the subtribe Cyrtopodiinae of the large pan-tropical tribe Cymbidieae. Linder & Kurzweil (1999) similarly consider *Eulophia* and *Acrolophia* to be closely related although Dressler (1993) placed these two genera in separate subtribes. The most recent revision of the southern African species of *Eulophia* was by Hall (1965).

The vegetative and floral morphology of *Eulophia* is similar to that of the well known genus *Cymbidium*. Growth is sympodial and each year a new vegetative and fertile shoot is produced from the previous year's pseudobulb or corm. Leaves are typically narrow and either conduplicate or plicate, although shade loving species have much broader, plicate leaves. Inflorescences are few to many flowered and are typically simple racemes although limited branching may occur in some species. Flowers may have small cryptically coloured sepals, or sepals identical to lateral petals. The column has a foot (mentum) in most species to which the labellum is attached. The labellum may be saccate or deeply spurred, although some species lack a spur. The column may be stout or slender with a terminal anther cap covering the two pollinia of the pollinarium. The two pollinia each have a cleft (Linder & Kurweil, 1999, consider these to be "four pollinia united in pairs") and are attached via elastoviscin threads to a thin-tissued stipe which is responsible for the bending reconfiguration in many species. The viscidium is typically triangular, with thick liquid glue. The stigma may be relatively exposed, or more commonly, is tucked behind the rostellum which may serve as a "scraper" to help with pollinium deposition (Dressler 1993).

The striking floral variation found among flowers in the genus hints at exciting evolutionary biology in which pollinators play a key role in diversification. However, the pollination biology of *Eulophia* has been largely undocumented. Of the approximately 230 species, only *Eulophia cristata*, pollinated by carpenter bees (Lock & Profita 1975) and several auto-

pollinating species from west Africa (Williamson 1984) have had their reproductive biology elucidated in any detail. A few additional observations exist in the literature of large *Xylocopa* carpenter bees visiting some of the large, showy species including *E. speciosa* (van der Cingel 2001) and *E. horsfallii* (Kullenberg 1961, Martins 2002). Dressler (1981) speculated that bees would be the most common pollinators in the subtribe Cyrtopodiinae which, in his 1981 classification, included *Eulophia* and *Acrolophia.*

In this study, I initially set out to identify pollinators of as many *Eulophia* species as possible and to map these onto a phylogeny of the genus to determine patterns of pollinator-driven radiation in this diverse orchid genus. The phylogeny reconstruction was thwarted by the lack of variation in the various nuclear (ITS) and plastid (psbA-trnH, matK) genomes that were sequenced. Despite this *Eulophia* has proven to be an intriguing study system shedding light on the ecology and evolution of deceptive pollination, mechanisms promoting cross-pollination, and pollinator-driven speciation.

Pollinators of the unusually pale blue-coloured *E. zeyheriana* were discovered foraging in the flowers of the rewarding plant *Whalenbergia cuspidata.* Given the superficial similarity to the human eye between the colours of the deceptive orchid and the rewarding *W. cuspidata*, I hypothesised that this system was a case of floral Batesian mimicry. If this is the case then flowers of the deceptive orchid should benefit by growing in proximity to the rewarding model. More generally the mimic would be expected to co-occur both in space (similar distribution) and time (common flowering phenology). In addition I expected the unusual colour of the *E. zeyheriana* flowers to be a critical component of this species' mimicry of *W. cuspidata.*

Early in this research I observed the rapid pollinarium bending in *Eulophia streptopetala.* I expected such bending reconfigurations to be widespread in the genus, although I also expected the timing of such reconfiguration to vary substantially given the diverse behaviour of the beetle and bee pollinators observed (sensu Darwin 1867).

All the species of *Eulophia* examined are deceptive. The discovery of nectar rewards in the flowers of A*crolophia cochlearis*, a member of a supposedly closely related genus, was therefore unexpected. The presence of a nectar reward was hypothesised to increase pollinator fidelity in this species and, as a result, I expected that pollen transfer efficiency would be higher in this species than in other deceptive *Acrolophia* species and the deceptive species of *Eulophia.*

Nectar rewards can however have negative consequences if pollinators spend extended periods visiting the plant. I therefore expected this species to have relatively high rates of pollinator-mediated self-pollination, but that this might be mitigated by mechanisms such as pollinarium reconfiguration that can protect against selfing.

While investigating the pollination of *Eulophia paviflora*, it became apparent that there were two morphologically distinct floral forms of this species, one short-spurred and the other long-spurred. It was therefore hypothesized that this represented a case of pollinator-driven divergence, in which each form had become specialised for pollination by a different insect pollinator or functional group of pollinators.

Using floral syndromes, I hypothesised that bee pollination systems would be frequent in this genus. Traits consistent with the bee pollination syndrome that are found in South African species of *Eulophia* include deep, zygomorphic, mechanically strong flowers with a "landing platform". Flowers are typically brightly coloured with fresh but not strong odours and have concealed sex organs.

In addition a number of *Eulophia* species have small flowers that do not open or open only briefly. The flowers of these species appeared to experience high rates of capsule set and I hypothesised that these species undergo autonomous self-pollination.

AIMS

My overall aim in this study was to further understanding of the ecology and evolution of orchids with deceptive pollination systems. Specific aims pertinent to the hypotheses outlined above were as follows:

Floral deception and mimicry

- To determine if the flower colour of the putative mimic, *E. zeyheriana*, is more similar to that of the proposed model *Wahlenbergia cuspidata* than it is to other species of *Eulophia* [chapter 2].

- To determine if flower colour affects fitness of the putative mimic *E. zeyheriana* [chapter 2].

- To quantify the importance of proximity of *E. zeyheriana* plants to rewarding model species for pollination success of these orchids [chapter 2].

- To investigate whether the distribution range and flowering phenology of the putative mimic *E. zeyheriana* are similar to those of its putative model *W. cuspidata* [chapter 2].

Cross-pollination mechanisms

- To document the incidence of pollinarium reconfiguration and survey the various modes of reconfiguration in the genus [chapters 2, 3, 4, 5, 6, 7].

- To test Darwin's cross-pollination hypothesis by determining whether pollinarium reconfiguration times show a positive relationship with pollinator visit times and generally exceed the latter [chapters 2, 3, 4, 5, 6, 7].

- To compare pollen transfer efficiency in the rewarding species *Acrolophia cochlearis* with deceptive congeners and a number of the deceptive species of *Eulophia* [chapter 5].

- To investigate rates of geitonogamous self-pollination in the rewarding species *A. cochlearis* using a new method for estimating rates of self- versus cross-pollination [chapter 5].

Pollinator-driven divergence

- To document the morphological, scent chemistry, phonological and distribution differences between two putative pollination-ecotypes of *Eulophia parviflora* [chapter 6].

- To determine if these differences are correlated with the pollinators of the two putative ecotypes and whether or not the pollinators can distinguish between the two ecotypes [chapter 6].

Case studies

- To describe the incidence and modes of insect pollination in the genus *Eulophia* [chapter 7].

- To describe the incidence and modes of autonomous self-pollination in the genus *Eulophia* [chapter 8].

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Floral deception and mimicry

[chapter 2]

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MIMICS AND MAGNETS: THE IMPORTANCE OF COLOR AND ECOLOGICAL FACILITATION IN FLORAL DECEPTION

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Abstract. Plants that lack floral rewards can attract pollinators if they share attractive floral signals with rewarding plants. These deceptive plants should benefit from flowering in close proximity to such rewarding plants, because pollinators are locally conditioned on floral signals of the rewarding plants (mimic effect) and because pollinators are more abundant close to rewarding plants (magnet effect). We tested these ideas using the non-rewarding South African plant *Eulophia zeyheriana* (Orchidaceae) as a study system. Field observations revealed that *E. zeyheriana* is pollinated solely by solitary bees belonging to a single species of *Lipotriches* (Halictidae) that appears to be closely associated with the flowers of *Wahlenbergia cuspidata* (Campanulaceae), a rewarding plant with which the orchid is often sympatric. The pale blue color of the flowers of *E. zeyheriana* differs strongly from flowers of its congeners, but is very similar to that of flowers of *W. cuspidata*. Analysis of spectral reflectance patterns using a bee vision model showed that bees are unlikely to be able to distinguish the two species in terms of flower color. A UV-absorbing sunscreen was applied to the flowers of the orchid in order to alter their color, and this resulted in a significant decline in pollinator visits, thus indicating the importance of flower color for attraction of *Lipotriches* bees. Pollination success in the orchid was strongly affected by proximity to patches of *W. cuspidata*. This was evident from one of two surveys of natural populations of the orchid, as well as experiments in which we translocated inflorescences of the orchid either into patches of *W. cuspidata* or 40 m outside such patches. Flower color and location of *E. zeyheriana* plants relative to rewarding magnet patches are therefore key components of the exploitation by this orchid of the relationship between *W. cuspidata* and *Lipotriches* bee pollinators.

Key words: *Batesian mimicry; bee vision;* Eulophia zeyheriana; *facilitation;* Lipotriches; *magnet species; Orchidaceae; pollination success; UV;* Wahlenbergia cuspidata.

INTRODUCTION

Deceptive pollination systems are known from at least 32 angiosperm families (Renner 2006). However, the vast majority of deceptive species belong to the Orchidaceae, a very large family in which ~30% of species lack floral rewards (van der Pijl and Dodson 1966). The most common deceptive systems in orchids involve the exploitation of food-seeking animals, and these have been considered to operate either through mimicry of specific rewarding flowers (Batesian floral mimicry) or nonspecific resemblance to rewarding flowers (generalized food deception; Jersáková et al. 2006).

Batesian floral mimics tend to exploit relatively specialized mutualisms between rewarding plants and their pollinators (Bronstein 2001, Anderson et al. 2005). This can require rather precise mimicry of the size (Galizia et al. 2005), shape (Johnson et al. 2003*a*), and color (e.g., Nilsson 1983, Johnson 1994, 2000, Anderson et al. 2005) of the floral display of rewarding plants. Roy and Raguso (1997) suggest that in a floral mimicry system, visual signals such as inflorescence color, size, and shape may be more important than scent, at least for bee pollinators. Recently, Galizia et al. (2005) examined the importance of scent and color in the Batesian mimicry system involving *Bellevalia flexuosa* (model) and *Orchis israelitica* (mimic). They found no evidence for scent mimicry in this system and point to visual similarity as being the key to the successful deception by *O. israelitica*.

Close matching of reflectance spectra between the flowers of Batesian mimics and their models has been demonstrated in several studies (Nilsson 1983, Johnson 1994, 2000, Johnson et al. 2003*a*, Anderson et al. 2005). It is often claimed that this similarity is adaptive, yet most studies fail to show that a mimic is more similar in color to a model than are conspecifics of the mimic. In one study, indirect evidence for adaptation was obtained from evidence that intraspecific variation in flower color of a Batesian mimic was correlated with the color of model flowers (Johnson 1994). Johnson et al. (2003*a*) furthermore argued that the cream color of a putative orchid mimic is likely to be adaptive because this trait is evolutionarily derived in its phylogenetic context.

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Galizia et al. (2005) showed that the flowers of a putative Batesian mimic are more similar in color to those of the model than are flowers of this orchid's nearest relatives.

Mimics may require not only overall similarity in flower color to their models, but also specific color patterning. Mimics that exploit mutualisms in which bees collect both nectar and pollen from flowers may need to imitate pollen rewards of their models. Nilsson (1983), for example, suggested that cream patches on the flowers of *Cephalanthera rubra* imitate pollen on the anthers of *Campanula* flowers. Heuschen et al. (2005) show that yellow UV-absorbing patches on flowers of many plants are attractive to pollen-collecting bees. They suggest that this is because these patches have a generalized resemblance to pollen.

There is increasing recognition that facilitation is an important ecological process in plant pollination systems (Feinsinger 1987, Callaway 1995, Feldman et al. 2004). The two main mechanisms of facilitation suggested by Moeller (2004), "joint attraction of pollinators" and "joint maintenance of pollinators," are bidirectional in the sense that facilitation benefits all of the interacting plants and apply mainly to situations in which all interacting species produce floral rewards. Unidirectional facilitation in pollination systems, on the other hand, is exemplified by the "magnet species effect" and "Batesian floral mimicry." In the magnet species effect (Thomson 1978), a rewarding "magnet" plant species increases local pollinator abundance in an area to the benefit of other plant species that have inferior rewards or lack rewards altogether (cf. Laverty 1992, Johnson et al. 2003*b*). In Batesian mimicry systems, deceptive mimics benefit from rewarding model species because these models condition pollinators to visit flowers of the mimic (cf. Nilsson 1983, Johnson 1994, 2000, Anderson et al. 2005). Some pollination systems apparently contain elements of both the magnet effect and Batesian floral mimicry. For example, pollination of the deceptive orchid *Anacamptis* (*Orchis*) *morio* is enhanced both by proximity to rewarding plants and the similarity of flower color to that of potential magnet plants (Johnson et al. 2003*b*). Gumbert and Kunze (2001) similarly showed that pollinators were more likely to approach flowers of the deceptive orchid *Anacamptis* (*Orchis*) *boryi* when foraging on rewarding flowers with similar colors to those of the orchid, suggesting a case of generalized Batesian floral mimicry. This is consistent with evidence that pollinators are more likely to switch foraging between similarly colored species (Chittka et al. 1997, Chittka and Raine 2006).

Johnson et al. (2003*b*) proposed that there may be a subtle continuum between generalized food deception and floral Batesian mimicry. This continuum may provide an evolutionary pathway, whereby natural selection might favor floral phenotypes of the deceptive species that resemble a co-occurring rewarding species, particularly if that rewarding species also facilitates pollination through a magnet effect. This could lead to a fine-tuned mimicry of the rewarding species in terms of morphology and color and perhaps also scent. However, there are still few studies of ecological facilitation in the pollination of deceptive plants (Dafni and Ivri 1979, Johnson 1994, Alexandersson and Ågren 1996, Gumbert and Kunze 2001, Johnson et al. 2003*b*), and we are aware of only one previous study in which the floral phenotype of a putative floral mimic has been manipulated in order to test its importance for pollination (Johnson et al. 2003*a*).

While searching for pollinators of the non-rewarding terrestrial plant *Eulophia zeyheriana* (Orchidaceae), we noticed that many of the bees sleeping in the pale blue flowers of a sympatric rewarding species, *Wahlenbergia cuspidata* (Campanulaceae), carried pollinaria of the orchid. The similarity in color of *E. zeyheriana* and *W. cuspidata* flowers and the fact that bees apparently visit both species led us to hypothesize that *E. zeyheriana* is to some degree a Batesian mimic of the flowers of *W. cuspidata*.

To test the idea that *E. zeyheriana* is a specialized exploiter of the relationship between *W. cuspidata* and its bee pollinators, we first asked whether the pollination success of *E. zeyheriana* was positively influenced by the local presence of *W. cuspidata* plants. We then investigated whether color of *E. zeyheriana* is more similar to that of its putative model in terms of the bee visual system than is the case for congeners of the orchid and whether manipulation of the flower color would influence visitation by bees. We also examined whether *E. zeyheriana* and *W. cuspidata* have similar distribution ranges, habitats, and phenology.

Material and Methods

The study species

Eulophia zeyheriana (Orchidaceae; Fig. 1A–C), the putative mimic, is restricted to the Drakensberg Mountains of the summer rainfall regions of South Africa (Appendix A: Fig. A1). The small, pale-blue flowers are borne on sparse, slender inflorescences reaching 20 cm in height. The pollinaria of this genus consists of two hard, solid pollinia and a stipe that undergoes rapid reconfiguration following removal of the pollinaria by pollinators. This correctly orients the pollinia to be deposited on the stigmas of flowers on subsequent visits and likely protects against geitonogamous pollen transfer (Peter and Johnson 2006*b*). Like all *Eulophia* species, this species is deceptive, and no nectar was discovered in any of the many flowers inspected.

Wahlenbergia cuspidata (Campanulaceae), the putative model, grows in clumps (Fig. 1D) at higher altitudes in the eastern parts of South Africa (Appendix A: Fig. A1). The pale-blue flowers are strongly protandrous. The anthers deposit pollen along the style that functions as a pollen presenter (Fig. 1E). After a period of about two days and following the removal of the majority of pollen

FIG. 1. (A) *Eulophia zeyheriana* (Orchidaceae) forms scattered groups in short grassland. (B) The small, pale blue flowers are arranged in (C) lax inflorescences. (D) *Wahlenbergia cuspidata* (Campanulaceae) grows in scattered clumps. (E) Flowers are at first male, with pollen exposed on the outside of the style that forms a pollen presenter. (F) After a period of 2–3 days the pollen has been removed and the tips of the pollen presenter flare open to expose the lobes of the stigma. (G) It appears that *Lipotriches* bees frequently visit the deceptive *Eulophia zeyheriana* flowers and most male *Lipotriches* bees bear pollinaria between their antennae. (H) The bees alight on the flowers and position themselves on the pollen presenter/style to probe the nectaries at the base of the petals. Scale bars: (A) 10 mm; (B, C, E–H) 5 mm; (D) 40 mm.

by pollinators, the stigma unfolds, becomes receptive, and the flower enters a female phase (Fig. 1F). Preliminary observations of single male- and female-phase flowers showed that small amounts of nectar are produced in concealed nectaries at the base of the pollen presenter/gynoecium (nectar volume [male phase, 0.46 μL; female phase, 1.76 μL], concentration [male phase, 25.70%; female phase, 27.33%], sucrose : hexose : fructose ratio [male phase, 44:56:0; female phase, 50:50:0]). These measurements were made from unbagged flowers at 09:00 and represent the standing crop of nectar available to bees. The flowers of this species rapidly close and nod over on the approach of inclement weather or in the evening, apparently in response to changing light conditions. This mechanism may serve to protect the pollen from water damage.

Study sites

Work was conducted primarily on the summit of Mount Gilboa at the extreme southwest of the Karkloof mountain range in central KwaZulu-Natal in eastern South Africa (Appendix A: Fig. A1). The Karkloof range is separated from the escarpment of Drakensberg Mountains by ~80 km. The area is burnt during the austral winter (primarily June–August) in most years. The vegetation of the summit of Mount Gilboa is an exceptionally diverse grassland community (~400 plant species). Following winter fires, the vegetation during the rainy summer months is characterized by short grass and numerous plants blooming en masse. At this site, *E. zeyheriana* plants are widely scattered over the summit and intermingled with more discrete clumps of *W. cuspidata* plants. The second study site was at Cobham, along the banks of the Pholela river in the foothills of the Drakensberg (Appendix A: Fig. A1). Here, the vegetation was dominated by relatively mature grasses and scattered plants of *E. zeyheriana*, while *W. cuspidata* plants were mainly found growing along footpaths. Few other species were in flower. At a large spatial scale at both sites, the orchids outnumbered *W. cuspidata* clumps by ~2:1, although at smaller spatial scales in the vicinity of *W. cuspidata* clumps, individual flowers of the orchid were usually outnumbered by those of *W. cuspidata*. Fieldwork was conducted between December 2001 and January 2002 as well as January and February of 2004.

Distribution and flowering phenology

Distribution and flowering phenology data were collected over the course of this study and supplemented with data from specimens housed in the Natal Herbarium (NH), University of KwaZulu-Natal Herbarium (NU), Schonland Herbarium, Grahamstown (GRA), and the Pretoria Herbarium (PRE). Flowering dates of herbarium specimens and our observations were renumbered, with 1 July being the first day of the season and 30 June the last day to span the austral summer. The distributions of renumbered flowering dates for *E. zeyheriana* and *W. cuspidata* were compared using the Kolmogorov-Smirnov test.

Pollinators

Bees were collected from flowers of *W. cuspidata* at Mount Gilboa and Cobham. Bees were either found sleeping in closed flowers in the late afternoon or during cloudy weather or foraging during sunny weather. Collected bees were identified by a specialist entomologist (C. Eardley, Department of Agriculture, South Africa), and voucher specimens were deposited in the Albany Museum, Grahamstown. Granular pollen loads on the bees were examined using Beattie's (1971) technique. Pollinaria of *E. zeyheriana* are easily distinguished by their size from those of co-occuring congeners.

We conducted 30 h of observations at Cobham, 35 h at the Mount Gilboa site, and 14 h at other sites in the Drakensberg Mountains. These observations span from mid-morning to late afternoon. Observation focused on patches of *W. cuspidata* and involve catching bees or observing their activity.

Survey of pollination success and translocation experiment

To determine the pollination success of *E. zeyheriana* and its relationship to the proximity of *W. cuspidata* plants, we recorded the proportion of flowers on each inflorescence that had pollinaria removed and those with pollinia deposited on their stigmas, as well as the distance to the nearest clump of *W. cuspidata*. We also determined the overall proportion of flowers on an inflorescence showing evidence of visitation (pollinarium removal and/or pollinia deposition). At Gilboa this included scoring flowers with only their anther caps removed, representing a failed visit. The first survey was conducted at Cobham during February 2002, and we scored the visitation rates and distance to the nearest *W. cuspidata* plant for all orchids encountered. A second survey was conducted on Mount Gilboa in February 2004. Due to the larger number of *W. cuspidata* and *E. zeyheriana* plants on Mount Gilboa, six transects were laid out, each at a random bearing from a different focal clump of *W. cuspidata*. Orchids within 1 m of the transect line were scored for visitation and distance from the *W. cuspidata* clump. Proportional visitation data were arcsine square-root transformed and related to the natural log of the distance to *W. cuspidata* patches using simple linear regression.

To complement these analyses based on natural distributions, we performed a translocation experiment at Mount Gilboa. *Eulophia zeyheriana* inflorescences were collected and visited flowers were removed before being placed in water containing glass pill vials taped to stakes and positioned in pairs either within patches of *W. cuspidata* or 40 m outside such patches. These were left for 9 d before being reexamined for signs of visitation. Proportion data were arcsine square-root

transformed and compared using a two-tailed paired t test.

Reflectance spectra

The reflectance spectra of various floral parts of the two species were measured using an Ocean Optics S2000 spectrophotometer (Ocean Optics, Dunedin, Florida, USA), coupled to an Ocean Optics Mini–D2T light source as described in Johnson et al. (2003*a*). We measured spectra of adaxial petal surfaces for both *E. zeyheriana* and *W. cuspidata* flowers, as well as the prominent white papillose area of the labellum of *E. zeyheriana* and the pollen-covered pollen presenter of male-phase *W. cuspidata* flowers. We also measured the reflectance spectra of 19 other species of *Eulophia* including all the likely sister taxa of *E. zeyheriana* (Hall 1965). Where more than one individual of each of these other species were measured, the average locus for the species was calculated in the color space.

Measured spectra were analyzed using the Chittka model to derive color loci in the bee color space (Chittka 1992, Chittka and Kevan 2005). This model uses the spectral sensitivity of honey bee receptors to calculate color loci. The sensitivities of Hymenoptera are phylogenetically conservative (Briscoe and Chittka 2001), and it is likely that the *Lipotriches* (Halictidae) bees involved in this pollination system have similar receptor sensitivities to honey bees. Perceptual color distances between average color loci were calculated using the equations of Chittka (1992). It seems likely that bumble bees (Dyer and Chittka 2004*a*, *b*) and honey bees (Giurfa 2004) can distinguish color loci with distances down to ~0.06 units apart, depending upon the color involved.

To determine whether flowers of the two species possess patterning in the UV region of the spectrum, flowers were photographed with a B + W 403 black filter (Jos. Schneider Optische Werke, Bad Kreuznach, Germany) that removes all wavelengths of light above 400 nm. Konica 400 ISO black and white film (Konica-Minolta, Tokyo, Japan) was used as it is sensitive to near-UV (~350–400 nm), but required exposures of ~90 s. The gray scale of Kevan et al. (1973) was used to judge the exposures.

Color modifications

To investigate the importance of color for pollinator attraction in this mimicry system, the reflectance of the adaxial and abaxial surfaces of the lateral petals of *E. zeyheriana* flowers were changed by painting them with a UV-absorbent mixture. This consisted of Parsol 1789 and Parsol MCX (Roche, Basel, Switzerland) dissolved in duck preen gland fat developed for modifying the reflective properties of birds feathers (Andersson and Amundsen 1997) and used by Johnson and Andersson (2002) to modify the color of *Hypoxis* flowers. Control flowers included flowers painted only with the preen fat and those with no treatment. Control flowers had a spot of the UV absorbing mixture applied behind the flower bract as a precaution to control for any potential odor of the active compounds, even though the active compounds are not volatile (Andersson and Amundsen 1997). These treatments were applied to plants growing in small groups at the Mount Gilboa site. After four days the flowers were scored for visitation. Transformed data were analyzed using an ANOVA. The effects of these manipulations on flower color were assessed using UV photography and analysis of reflectance spectra using the model of Chittka (1992).

Breeding systems

Inflorescences were bagged and virgin flowers were either self-pollinated, outcrossed with pollinia from other individuals growing more than 10 m away, or left untreated to test for autogamy. In most cases, one of each of these three treatments was applied to each inflorescence.

Results

Distributions and phenology

The distributions of *E. zeyheriana* and *W. cuspidata* overlap broadly and both are confined to grasslands at higher altitudes in the eastern parts of South Africa (Appendix A: Fig. A1). At six separate sites we have visited, the two species co-occur. Therefore, the apparent absence of *Wahlenbergia* from some sites where the orchid has been collected (Appendix A: Fig. A1) likely reflects under-collecting of herbarium specimens rather than true incongruence. Flowering of *E. zeyheriana* overlaps broadly with *W. cuspidata* (Appendix B: Fig. B1). While flowering of *E. zeyheriana* peaks ~10 days earlier than *W. cuspidata*, at both sites there is no significant difference in the distribution of flowering times of the two species (Kolmogorov-Smirnov test, $P > 0.10$ for Gilboa, $P > 0.05$ for Cobham).

Pollinators

In total, we observed more than 70 individual bees bearing pollinaria of *E. zeyheriana* and caught 46 of these bees. All belonged to a single *Lipotriches* species (Halictidae), the identity of which remains unresolved due to the uncertain taxonomy of this genus (C. Eardley, *personal communication*). During the 2001–2002 flowering season, we caught 11 *Lipotriches* bees bearing *E. zeyheriana* pollinaria or viscidia between their antennae (Fig. 1G) on Mount Gilboa and nine at Cobham. All bees were either roosting in *W. cuspidata* flowers or foraging from the concealed nectaries at the base of the flowers (Fig. 1H). This bee was the only insect species observed to visit the flowers of *W. cuspidata*. In January and February 2004, ~50 *Lipotriches* bees bearing pollinaria were observed in *W. cuspidata* flowers on Mount Gilboa and 26 of these bees were captured. At the Cobham site, 79% of the bees collected bore at least one pollinarium or viscidium, while 59% of bees collected or observed at Gilboa carried pollinaria or viscidia. A number of bees carried more than one

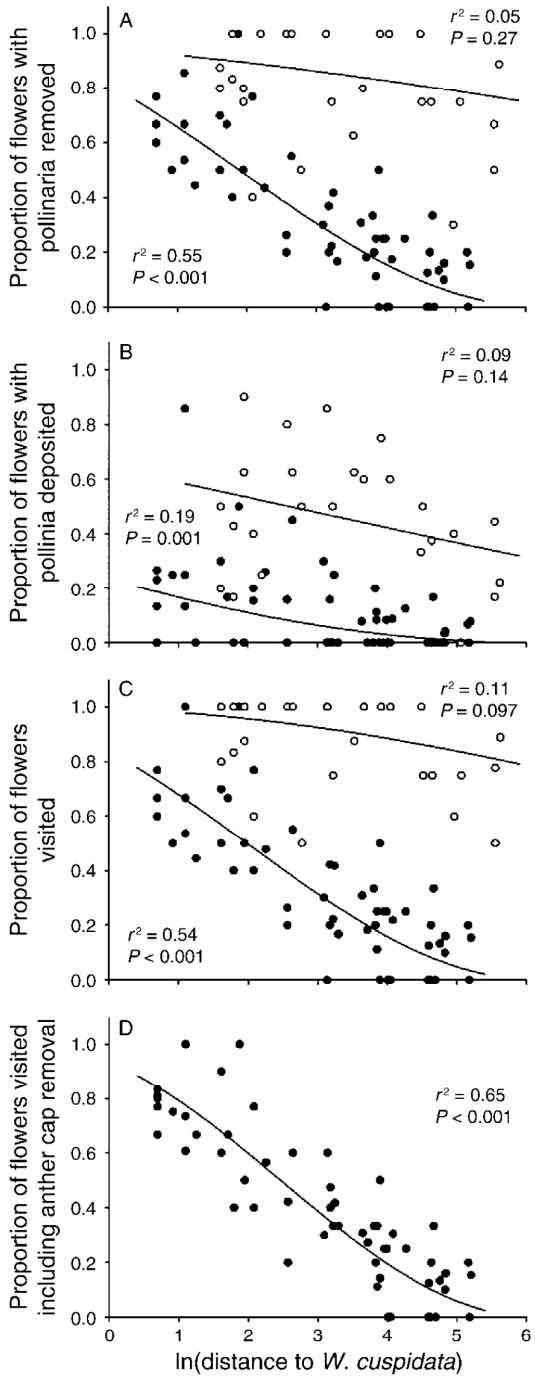

FIG. 2. Pollination success of *Eulophia zeyheriana* at increasing distances from patches of *Wahlenbergia cuspidata* plants at Cobham (open circles) and Mount Gilboa (solid circles). This includes the proportion of flowers on an inflorescence (A) with pollinaria removed, (B) with pollinia deposited, (C) with signs of either pollen removal or deposition, and (D) showing any sign of visitation, including those flowers with their anther caps dislodged. Proportion data were arcsine square-root transformed, and distance data were natural log-transformed for regression analysis. Distances were measured in meters. The back-transformed proportion data and regression lines are presented.

pollinarium, one individual bearing four viscidia as well as a complete pollinarium. Of the 218 *E. zeyheriana* flowers found to have pollinia deposited on their stigmas, 58% contained a single pollinium, 40% contained two pollinia, three stigmas had three pollinia deposited, and a single flower had four pollinia deposited on its stigma. We observed two direct visits to flowers of *E. zeyheriana* in 2004. Each visit lasted 20–25 s and entailed the bee alighting on the flower, positioning itself on the labellum, and probing around base of the labellum and column, apparently inspecting the spur for nectar. In one case, a bee deposited pollinia it was carrying and removed a second pollinarium.

All of the 46 *Lipotriches* bees collected over two flowering seasons at both sites were male. This includes bees sleeping in *W. cuspidata* flowers, bees foraging on the *W. cuspidata* flowers, and the two bees collected following their visits to *E. zeyheriana* flowers.

Although granular pollen of other taxa was represented on all bees, only *W. cuspidata* pollen was found on every bee inspected (Appendix C: Table C1). Bees from Mount Gilboa carried significantly more pollen grains of *W. cuspidata* than bees from Cobham ($t_{29} = 2.3$, $P = 0.0160$). They also had significantly more pollen grains from other taxa ($t_{29} = 3.8$, $P = 0.0003$), and significantly more taxa are represented in these pollen loads at Mount Gilboa ($t_{29} = 9.5$, $P < 0.0001$; Appendix C: Table C1). Other taxa well represented in the pollen loads of the bees inspected include species of Asteraceae and Fabaceae (Appendix C: Table C1).

Population survey and translocation experiments

Pollination success and overall rates of visitation in *E. zeyheriana* declined with increasing distance from focal *Wahlenbergia cuspidata* plants at both sites (Fig. 2), but these univariate relationships were significant only for the Mount Gilboa site (Fig. 2). Overall visitation success of *E. zeyheriana* was significantly greater at the Cobham site (ANCOVA, $F_{1,78} = 4.99$, $P = 0.028$; Fig. 2C).

A similar trend of decreased pollination success with increased distance from *W. cuspidata* plants was revealed by the translocation experiment. Measures of pollinaria removal and overall visitation were both significantly lower in inflorescences that were translocated to a position 40 m from the nearest *W. cuspidata* plant compared to those translocated within a clump of *W. cuspidata* (Fig. 3). Pollinia deposition was also lower in plants translocated outside of *W. cuspidata* clumps, but not significantly so.

Reflectance spectra

Flowers of both *E. zeyheriana* and *W. cuspidata* appear pale blue to humans with pale blue to white pollen presenters or labellae (Figs. 1 and 4A, C). However, in the near-UV region, the "white" papilose area of the rolled labellum of *E. zeyheriana* and the pollen presenter of *W. cuspidata* appear strongly UV absorbent (Fig. 4B, D).

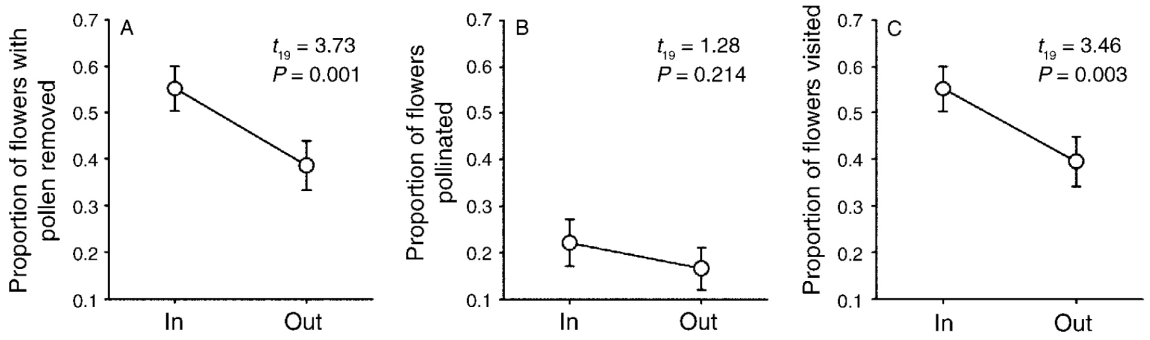

FIG. 3. Pollination success (mean ± SE) of *Eulophia zeyheriana* inflorescences translocated into patches of *Wahlenbergia cuspidata* plants (in) and inflorescences translocated 40 m from patches of *W. cuspidata* (out). This includes the proportion of flowers on an inflorescence (A) with pollinaria removed, (B) with pollinia deposited, and (C) with signs of either pollen removal or deposition. Proportion data were arcsine square-root transformed. Two-tailed *P* values were determined using a paired *t* test ($n = 20$ pairs of inflorescences).

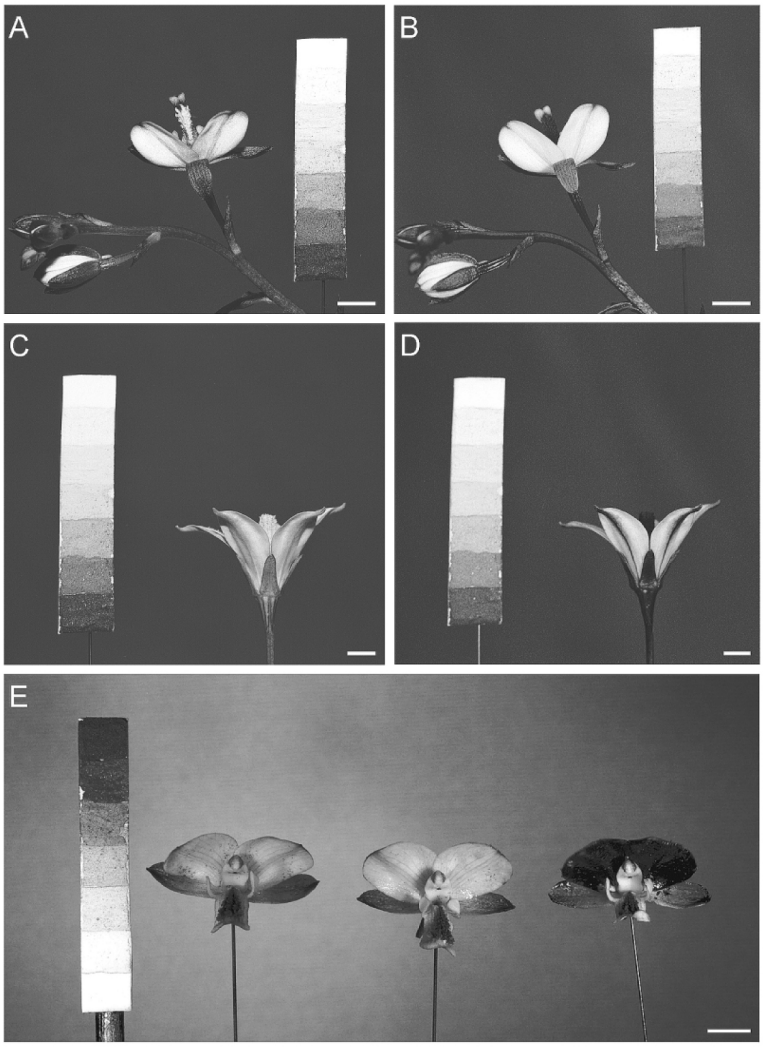

FIG. 4. Appearance of (A) *Eulophia zeyheriana* and (C) *Wahlenbergia cuspidata* flowers in the human visual spectrum. Appearance of (B) *E. zeyheriana* and (D) *W. cuspidata* flowers in the near-UV region. (E) Appearance of *E. zeyheriana* flowers with a UV-absorbent mixture (right) and with a control mixture (middle) in the near-UV region; the leftmost flower is an untreated control. Scale bars in each panel indicate 5 mm.

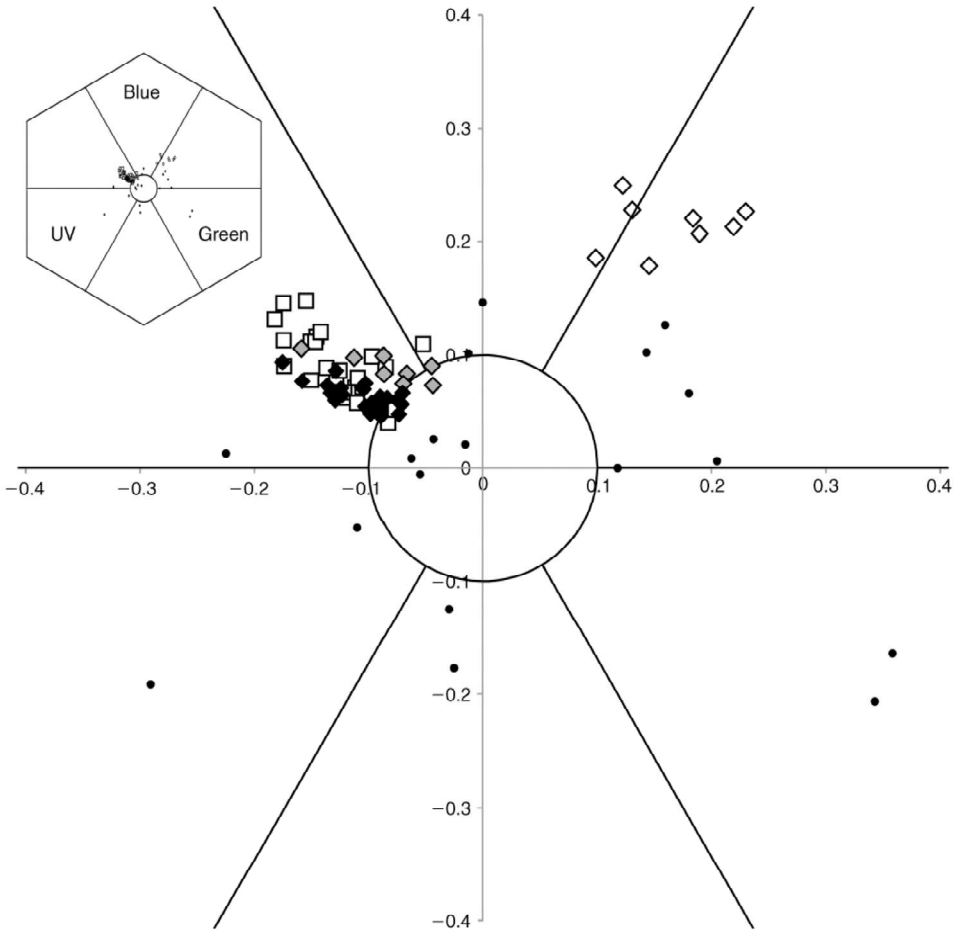

FIG. 5. Color loci of adaxial petal surfaces of individuals of *Eulophia zeyheriana* (solid diamonds) and *Wahlenbergia cuspidata* (open squares), as well as mean color loci for 19 other species of *Eulophia* (solid circles), calculated according to the Hexagon color model of bee vision (Chittka 1992). The color loci of *E. zeyheriana* petals modified by the addition of a UV-absorbent mixture (open diamonds) as well as *E. zeyheriana* petals coated with the control mixture (gray diamonds) are also shown. The inset shows segments and is labeled blue, green, and UV. Segments between these are combinations, e.g., blue-green, green-UV, UV-blue. The central circle indicates colors that are close to the green background and hence difficult for bees to perceive. The axes correspond to the excitation values of the two types of color opponent neurons (Chittka and Raine 2006).

Spectrophotometry confirmed these qualitative observations. The average reflective spectra of the petals of *E. zeyheriana* and *W. cuspidata* are similar and include reflectance in the near-UV region between 350 and 400 nm (Appendix D: Fig. D1A). The shape of the average reflectance curves is likewise similar when comparing the labellum of *E. zeyheriana* and the pollen presenter of *W. cuspidata*, although the overall brightness of the labellum of *E. zeyheriana* is higher and there is no UV reflectance from these parts of the flowers (Appendix D: Fig. D1B).

Analysis of individual spectra using the bee vision model of Chittka (1992) showed that adaxial petal colors fall in the blue-UV segment of the color hexagon and there is broad overlap of measurements made for *E. zeyheriana* and *W. cuspidata* (Fig. 5). The perceptual color distance between average loci of the two species is only 0.03 color opponent units. These colors are quite distinct from the average colors of a number of other southern African species of *Eulophia* including likely sister taxa (Fig. 5). The average distance of the color loci of the petals of different *Eulophia* species is 0.24 units, with a range of 0.09–0.56 units.

The color of *E. zeyheriana* labellae is similar to the pollen presenter of *W. cuspidata*, and there is some overlap of loci (Fig. 6), with a distance between the average loci of 0.06 units. A number of other species of *Eulophia* have similar-colored labellae with average loci grouping in the blue-green region of the color hexagon. The closest of these are two autogamous "varieties" of *Eulophia clavicornis* at 0.06 and 0.09 color opponent units. The average distance of the color loci of the labellae of different *Eulophia* species is 0.29 units, with a range of 0.06–0.69 units.

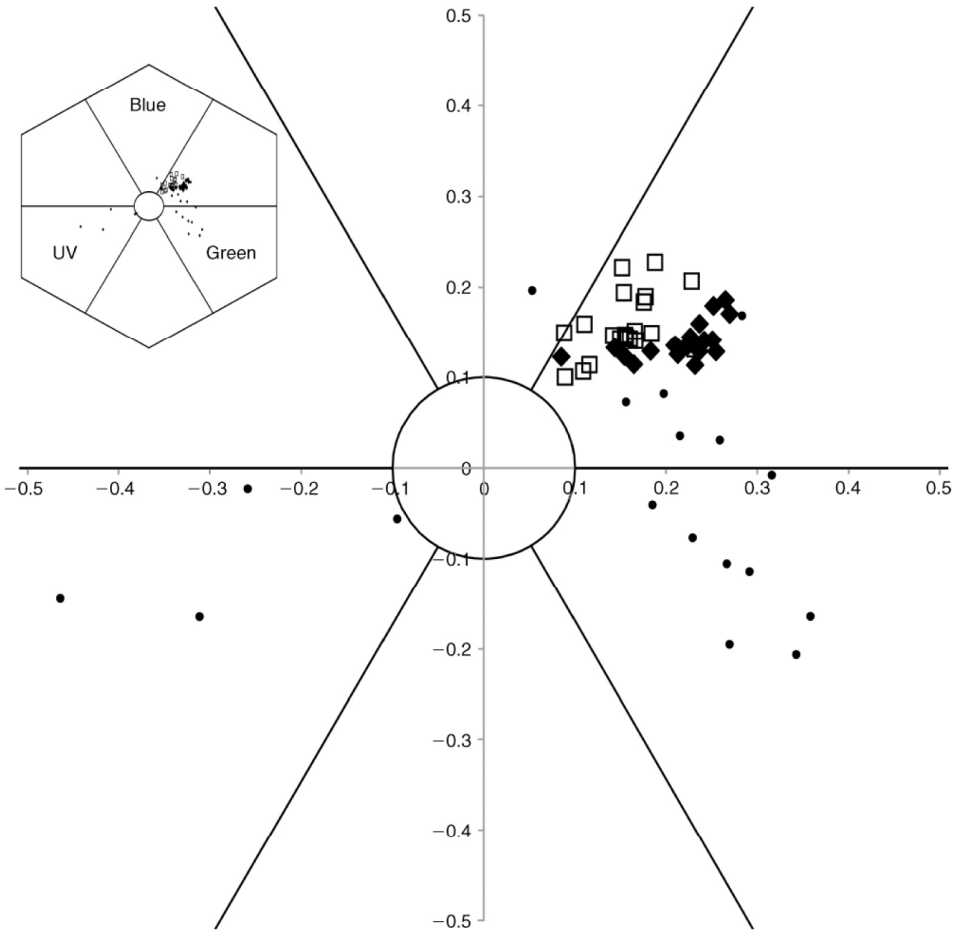

FIG. 6. Color loci of the labellae of *Eulophia zeyheriana* (solid diamonds) and pollen presenter of *Wahlenbergia cuspidata* (open squares). Mean color loci of the labellae of a number of other species of *Eulophia* are also included (solid circles). Color loci are calculated according to the Hexagon color model of bee vision (Chittka 1992). See Fig. 5 for additional details.

Color modifications

Qualitatively, the addition of the UV-absorbing treatment substantially alters the appearance of the lateral petals of the flower in the near-UV region of the spectrum (Fig. 4E) relative to the two controls. The manipulated control, painted with duck preen fat, appears slightly more reflective in the near-UV region when compared to the unmanipulated control (Fig. 4E). The colors of the manipulated controls are similar to those of unmanipulated *E. zeyheriana* petals and comparable to the petals of *W. cuspidata* with perceptual color distances of <0.04 units between average loci. In contrast, the colors of the petals treated with the UV-absorbing Parsol mixtures appear distinctly different from the average *W. cuspidata* and unmanipulated *E. zeyheriana* flowers (color opponent distance of 0.32 and 0.31 units, respectively) in the blue-green region of the color hexagon (Fig. 5).

Flowers with the UV-absorbent treatment applied had significantly lower rates of pollinarium removal and overall visitation than the treated and untreated controls. The two controls did not differ significantly from one another (Table 1). The UV-absorbent treatment also had lower rates of pollinia deposition than the two controls, although this was not significant.

Breeding systems

Eulophia zeyheriana is self-compatible, and capsules produced from selfing are comparable in mass to those resulting from outcrossing. However, the quality of seed resulting from self-pollination is significantly inferior to seed produced by outcrossing, with one-third fewer seeds with embryos per capsule (Appendix E: Table E1). While hand-pollination of flowers ($n = 33$) always resulted in fruit set, none of the bagged and unmanipulated flowers ($n = 16$) set fruit. This response to hand-pollination is significant ($G = 59.3$, $P < 0.0001$) and indicates that pollinator visits are required for fruit set in *E. zeyheriana*.

DISCUSSION

The results of this study indicate that the deceptive orchid *Eulophia zeyheriana* exploits a specialized inter-

TABLE 1. Pollination success of *Eulophia zeyheriana* flowers with modified UV reflectance.

Treatment	No. inflorescences	No. flowers	Proportion of pollinaria removed	Proportion of pollinia deposited	Proportion of flowers visited
Untreated control	16	43	0.30[a]	0.16	0.33[a]
Treated control	14	35	0.40[a]	0.09	0.43[a]
UV treatment	17	41	0.05[b]	0.05	0.05[b]
$F_{2,44}$		0.22	4.89	1.06	5.18
P		0.80	0.012	0.35	0.010

Notes: Values within a column that share the same letter are not significantly different (based on one-way ANOVA of inflorescence-level data, followed by Tukey multiple comparisons).

action between *Wahlenbergia cuspidata* and a single species of *Lipotriches* bee primarily by means of color signalling. Although flowers of the orchid differ morphologically from those of *W. cuspidata* (Fig. 1A–F), a large proportion of the bees caught carried pollen of both species, suggesting that they cannot easily distinguish the deceptive orchid from the rewarding *Wahlenbergia*.

The basis for the lack of discrimination by bees is probably that the color of the petals of the orchid is very similar to that of petals of the model in bee color space (Fig. 5), being only 0.03 units apart on average. There is evidence that bumble bees and honey bees cannot distinguish colors less than 0.06 units apart (Giurfa 2004, Dyer and Chittka 2004*a*, *b*). This is supported by our color modification treatments. The mean color distance of the manipulated controls was 0.03 units from the untreated flowers and 0.04 units from the *W. cuspidata* but the visitation rates to these flowers was not significantly different from that of the untreated flowers. In contrast, flowers that had the UV component of their flower colors experimentally removed (color opponent distance of 0.32 units to the model flowers) were effectively ignored by the pollinators and had very low visitation rates (Table 1).

By altering the color of the flowers, the mimicry is apparently disrupted in much the same way as altering the shape of inflorescences was shown to reduce visits by pollinators to a Batesian mimic in a fly-pollinated system (Johnson et al. 2003*a*). By contrast, we have found that manipulation of UV reflectance (using the same technique as described in this study) in the bee-pollinated generalized food deceptive orchid *Eulophia parviflora* actually resulted in significantly increased visitation (C. I. Peter and S. D. Johnson, *unpublished data*). Indeed, negative frequency-dependent selection on flower color has been predicted for generalized food deceptive systems (Smithson and Macnair 1997, Gigord et al. 2001, Lynn et al. 2005).

The phylogenetic relationships within the large genus *Eulophia* remains unresolved. However, *E. zeyheriana* is the only species in the genus with pale blue flowers having loci in the blue-UV segment of the bee color hexagon. This is in contrast to other congenerics with loci in the other five segments (Fig. 5). It is likely, therefore, that the floral coloration of *E. zeyheriana* is a derived trait in the phylogenetic sense (cf. Wanntorp 1983). This, considered together with the results of the spectral analyses and color manipulation experiments (Figs. 4 and 5, Table 1), suggests that the pale blue flower color in *E. zeyheriana* could be an adaptation for exploiting the relationship between *W. cuspidata* and its *Lipotriches* bee pollinator.

Besides the overall similarity of petal color between model and mimic, the labellum of *E. zeyheriana* may also mimic the pollen presenter of *W. cuspidata*. Heuschen et al. (2005) suggest that UV-absorbing patches on flowers may mimic the UV-absorbing signal of pollen, with UV absorption thought to be a result of flavonoid pigments that protect the pollen against bateria, fungi, and UV damage. Our data suggest a more specific case of pollen mimicry, with the white, UV-absorbing area on the labellum of *E. zeyheriana* mimicking the unusual pale blue to white pollen (and pollen presenter) of *W. cuspidata* (Fig. 1B, E, F). While the male bees that we observed would not be seeking out the pollen of *W. cuspidata* as a primary reward, the pollen presenter is visually a central feature of *W. cuspidata* flowers and is apparently used by many of the bees to orient in the flower to access nectar (Fig. 1H). Nilsson (1983) proposed a similar system with a patch of cream frills on the labellae of *Cephalanthera ruba* thought to mimic the pollen presenters of *Campanula* flowers.

Unlike the novel color of the petals of *E. zeyheriana*, the color of the pollen-mimicking patch on the labellum is similar to that found in closely related taxa. Similar yellow, UV-absorbing patches are found on labellae of a number of other species of *Eulophia* (Fig. 6, lower right segment) and may be an important component of generalized food deception in these species. This trait in *E. zeyheriana* is therefore most likely a preadaptation that has been enhanced by the rolling of the labellum to more closely resemble the pollen presenter of *W. cuspidata* (Figs. 1C and 5A, B).

Little is known about the biology of *Lipotriches* bees. Immelman and Eardley (2000) reported that some *Lipotriches* species collect grass pollen to provision their

nests. They found that female *Lipotriches* bees emerged between 06:30 and 07:30 to forage for pollen (and presumably also nectar from plants other than grasses) and then sealed themselves into their nest with mud plugs by midday. Tchuenguem Fohouo et al. (2004) documented nearly identical foraging times for another grass-pollen-collecting *Lipotriches* from west Africa. It is puzzling that only male bees were observed on *Wahlenbergia* flowers, but as our observations usually commenced only mid-morning, we cannot firmly exclude the possibility that *Wahlenbergia* flowers (and those of the orchid) are also visited by female bees.

Batesian floral mimicry

Eulophia zeyheriana is from a lineage of deceivers: none of the species we have examined in this genus have rewards and most *Eulophia* species are pollinated by naive insects through generalized food deception (Peter and Johnson 2006*a*; C. I. Peter, *unpublished data*). These can be considered generalized exploiters as they exploit generalist mutualisms between pollinators and suites of plants.

Eulophia zeyheriana, in contrast, appears to be specialized to exploit the very close association between *W. cuspidata* and a single species of *Lipotriches* bee. This orchid cannot set seed without pollinator visits (Appendix E: Table E1), and this bee was the only insect species observed to carry its pollinaria. These observations, together with the close spectral matching (Fig. 5) and facilitated pollination at one site and in the translocation experiment (Figs. 2 and 3), are consistent with the initial hypothesis of Batesian floral mimicry. The hypothesis is further supported by the geographical distribution of the orchid, which is broadly congruent with that of *W. cuspidata* (Appendix A: Fig. A1), and the presence of *W. cuspidata* plants at all of the six sites where we have observed the orchid. Flowering times of the two species are very similar, although the orchids sometimes flower a few days earlier than the rewarding model (Appendix B: Fig. B1). This and the observation that pollination of orchids in at least one population did not depend strongly on proximity to *W. cuspidata* plants (Fig. 2) suggest that the orchids may, under some circumstances, attract *Lipotriches* bees that have not already been conditioned by visiting *W. cuspidata* flowers.

Facilitated pollination

There is increasing evidence that interactions among coexisting plants can be characterized by facilitation, rather than competition (e.g., Callaway 1995). As a result of pollinator conditioning, Batesian floral mimicry systems should be characterized by unidirectional facilitation of pollination success in mimics by their models (Johnson 1994), although this special case has received little attention in earlier literature on ecological facilitation (e.g., Callaway 1995). Facilitation of pollination among co-occuring plant species is not confined to mimicry systems, however, and may be a more general phenomenon, as in the "magnet species effect" (Thomson 1978), whereby local aggregation of pollinators around particularly rewarding plants, rather than pollinator conditioning per se, enhances the pollination of other plants in the close vicinity (cf. Laverty 1992).

We found that pollinaria removal and overall visitation of *E. zeyheriana* was significantly enhanced by proximity to *W. cuspidata* plants in one population (Gilboa), while in another population (Cobham) we did not find a significant relationship between these variables (Fig. 2). One plausible explanation for the lack of a significant facilitation effect in the Cobham population is simply that the removal of pollinaria from almost 100% of flowers at this site (Fig. 2), presumably because bees were particularly abundant, makes it difficult to use pollinaria removal rates to assess variation in actual visitation rates among plants (Fig. 2C). Further evidence for facilitation was obtained in an experiment in which plants were translocated either in or out of patches of *W. cuspidata* at the Gilboa site. In this experiment, pollinaria removal and overall visitation, but not pollen deposition, were significantly affected by proximity to *W. cuspidata* patches (Fig. 3). Rates of pollinaria removal were much higher than rates of pollen deposition in this experiment, which suggests either that most bees that visited the experimental inflorescences were not already carrying pollinaria or that pollinium deposition, even by bees already carrying pollinaria, occurs during a smaller fraction of visits than does pollinarium removal.

Other studies of deceptive orchids have yielded mixed evidence for the idea that pollination can be facilitated by co-occuring rewarding plants (Johnson 1994, Lammi and Kuitunen 1995, Alexandersson and Ågren 1996, Johnson et al. 2003*b*, Juillet et al. 2007). Facilitation of pollination by a rewarding species was evident in several populations of the Batesian floral mimic *Disa ferruginea* (Johnson 1994) and the generalized food deceptive orchid *Anacamptis morio* (Johnson et al. 2003*b*), while it was detectable in only one of three years in a study of the generalized food deceptive orchid *Calypso bulbosa* (Alexandersson and Ågren 1996).

In none of the above studies including the present one can facilitation be firmly ascribed to either pollinator conditioning (mimicry effect) or pollinator abundance (magnet effect). While our color manipulation data indicates that the resemblance between the model and the mimic is important for deception of pollinators, we don't yet know whether the color preferences of the bees are innate or learned. Furthermore, there could also be a magnet effect behind the enhancement of *E. zeyheriana* pollination by *W. cuspidata* at the Gilboa site (Fig. 2A), as *Lipotriches* bees appear to be concentrated around patches of *W. cuspidata*. Further work is required to unravel the precise contributions of the mimic and magnet effects to the ecological facilitation of pollination.

Acknowledgments

We thank KZN Wildlife for access to their reserves, Connal Eardley for identifying pollinators, PRECIS for distribution and phenology data, Lars Chittka for help with the color model, and Greg Anderson, who caught the first bees bearing *E. zeyheriana* pollinaria. Susi Vetter, Brad Ripley, and two reviewers suggested improvements to the manuscript. The NRF and Rhodes University funded this study.

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APPENDIX A

A figure showing the distribution of *Eulophia zeyheriana* and *Wahlenbergia cuspidata* in eastern South Africa (*Ecological Archives* E089-094-A1).

APPENDIX B

A figure showing the overlap of flowering phenology of *Eulophia zeyheriana* with that of *Wahlenbergia cuspidata* (*Ecological Archives* E089-094-A2).

APPENDIX C

A table showing the mean pollen loads on *Lipotriches* bees at the Mount Gilboa and Cobham study sites (*Ecological Archives* E089-094-A3).

APPENDIX D

A figure showing the mean reflectance spectra of *Eulophia zeyheriana* and *Wahlenbergia cuspidata* flower parts (*Ecological Archives* E089-094-A4).

APPENDIX E

A table showing the results of a breeding system experiment for *Eulophia zeyheriana* (*Ecological Archives* E089-094-A5).

Craig I. Peter and Steven D. Johnson. 2008. Mimics and magnets: The importance of color and ecological facilitation in floral deception. *Ecology* 89:1583–1595.

Appendices

Appendix A: A figure showing the distribution of *Eulophia zeyheriana* and *Wahlenbergia cuspidata* in eastern South Africa.
***Ecological Archives* E089-094-A1.**

Appendix B: A figure showing the overlap of flowering phenology of *Eulophia zeyheriana* with that of *Wahlenbergia cuspidata*.
***Ecological Archives* E089-094-A2.**

Appendix C: A table showing the mean pollen loads on *Lipotriches* bees at the Mt. Gilboa and Cobham study sites.
***Ecological Archives* E089-094-A3.**

Appendix D: A figure showing the average reflectance spectra of *Eulophia zeyheriana* and *Wahlenbergia cuspidata* flower parts.
***Ecological Archives* E089-094-A4.**

Appendix E: A table showing the results of a breeding system experiment for *Eulophia zeyheriana*.
***Ecological Archives* E089-094-A5.**

Ecological Archives **E089-094-A1**

Craig I. Peter and Steven D. Johnson. 2008. Mimics and magnets: The importance of color and ecological facilitation in floral deception. *Ecology* 89:1583–1595.

Appendix A. A figure showing the distribution of *Eulophia zeyheriana* and *Wahlenbergia cuspidata* in eastern South Africa.

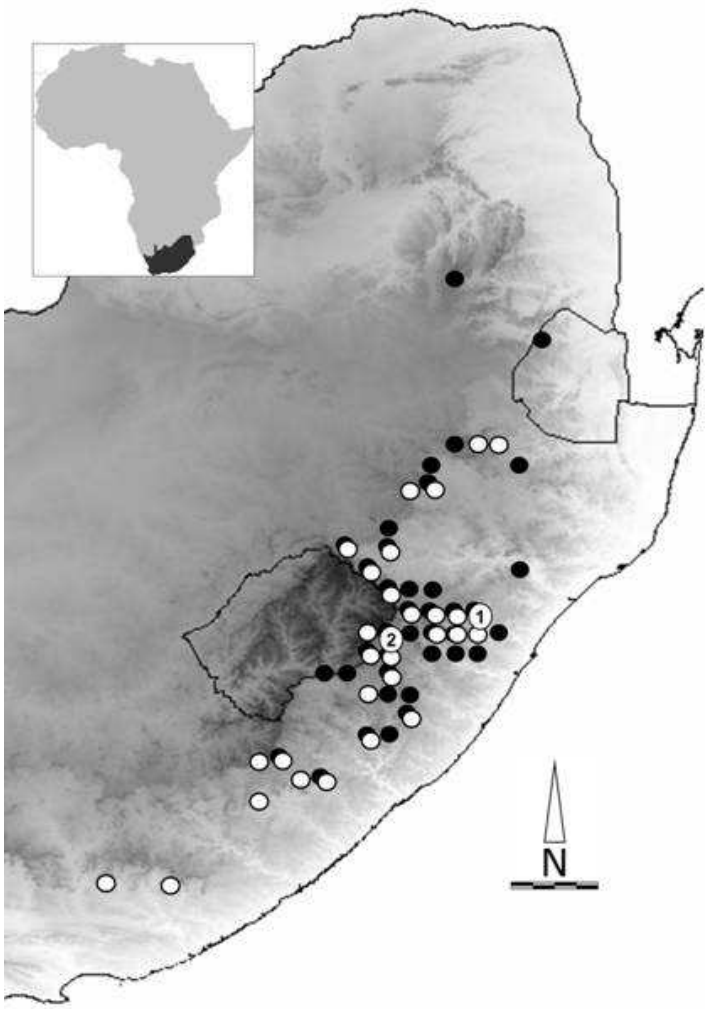

FIG. A1. Distribution of *E. zeyheriana* (●) and *W. cuspidata* (○) in the eastern parts of South Africa. Study site include (1) Mt. Gilboa and (2) Cobham. Each dot represents one or more herbarium specimen or field observations (where herbarium specimens could have been made at different localities and/or dates during the course of this study) occurring in a quarter degree square. Darker shades of gray represent increasing altitudes. Sacle bar: 100 km.

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Ecological Archives E089-094-A2

Craig I. Peter and Steven D. Johnson. 2008. Mimics and magnets: The importance of color and ecological facilitation in floral deception. *Ecology* 89:1583–1595.

Appendix B. A figure showing the overlap of flowering phenology of *Eulophia zeyheriana* with that of *Wahlenbergia cuspidata*.

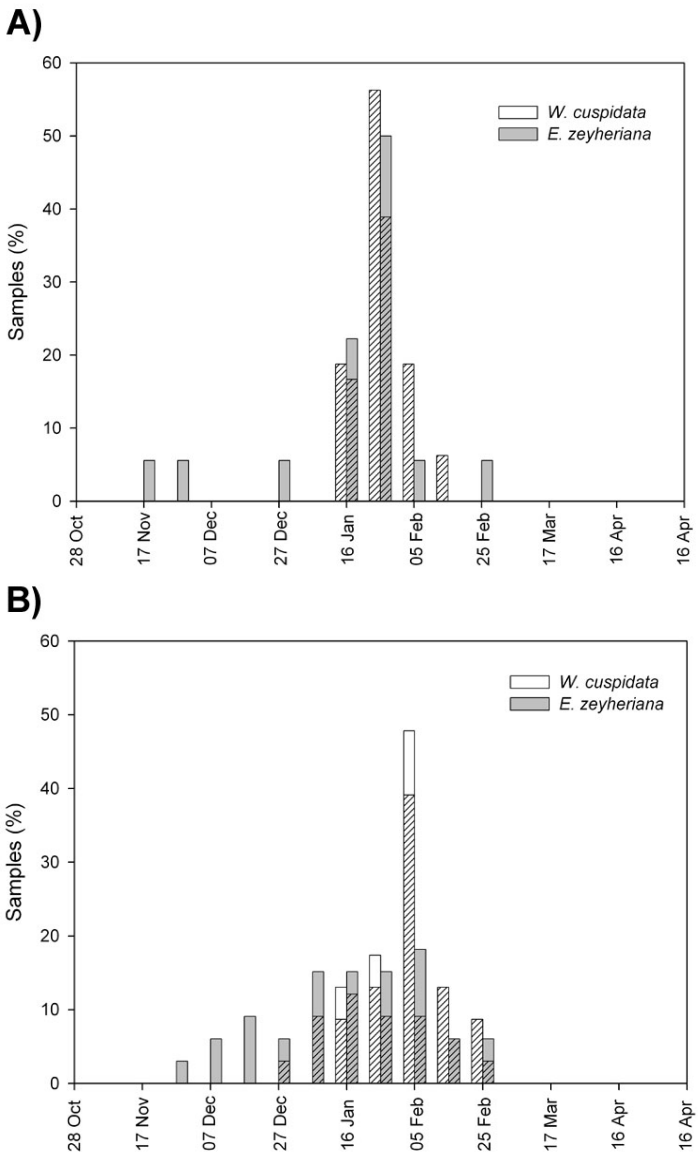

FIG. B1. Flowering phenology of *E. zeyheriana* overlaps broadly with that of *W. cuspidata* at both (A) the Mt. Gilboa site and (B) Cobham. Samples include herbarium specimens (unhatched; *E. zeyheriana*: n_{gilboa} = 8, n_{cobham} = 16; *W. cuspidata*: n_{gilboa} = 0, n_{cobham} = 4) and field observations where herbarium specimens could have been made (different localities and/or dates) during the course of this study (hatched; *E. zeyheriana*: n_{gilboa} = 10, n_{cobham} = 17; *W. cuspidata*: n_{gilboa} = 16, n_{cobham} = 19). Each bar represents a period of ten days. Median flowering dates for Gilboa are 18 June vs. 22 June for *E. zeyheriana* and *W. cuspidata* respectively. For Cobham, medain flowering dates are 13 June vs. 22 June for *E. zeyheriana* and *W. cuspidata* respectively.

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Ecological Archives E089-094-A3

Craig I. Peter and Steven D. Johnson. 2008. Mimics and magnets: The importance of color and ecological facilitation in floral deception. *Ecology* 89:1583–1595.

Appendix C. A table showing the mean pollen loads on *Lipotriches* bees at the Mt. Gilboa and Cobham study sites.

TABLE C1. Pollen loads on *Lipotriches* bees collected at the Mt. Gilboa and Cobham study sites.

	Gilboa (n = 15 bees)			Cobham (n = 15 bees)	
Taxon	Percentage of bees carrying	Average number of pollen grains per bee		Percentage of bees carrying	Average number of pollen grains per bee
CAMPANULACEAE					
Wahlenbergia cuspidata	100.0	16.3		100.0	84.9
Wahlenbergia cuspidata in faeces	0.0	0.0		33.3	6.3
Unidentified small pink "*Wahlenbergia*"	6.7	0.1		66.7	1.8
FABACEAE					
Psobubia	6.7	0.1		6.7	0.1
Lotonotis	13.3	0.4		86.7	38.5
Pearsonia	6.7	0.1		0.0	0.0
Unidentified sp. 1	6.7	1.1		0.0	0.0
ASTERACEAE					
Unidentified sp. 2	20.0	0.7		0.0	0.0
Unidentified sp. 3	60.0	2.9		80.0	4.5
Unidentified sp. 4	26.7	0.5		53.3	1.1
Unidentified sp. 5	0.0	0.0		6.7	1.1
Unidentified sp. 6	0.0	0.0		60.0	3.1
OTHER					
POACEAE	33.3	0.8		20.0	0.3
SCROPHULARIACEAE - *Cycnium*	0.0	0.0		40.0	3.3
IRIDACEAE - *Dierama*	0.0	0.0		20.0	0.6
APIACEAE - *Alepidea*	0.0	0.0		40.0	5.8
COMMELINACEAE - *Cyanotis*	6.7	0.1		0.0	0.0
Unidentified sp. 7	73.3	10.1		13.3	0.3
Unidentified sp. 7 faeces?	46.7	107.5		0.0	0.0
Unidentified sp. 8	13.3	0.1		0.0	0.0
Unidentified sp. 9	0.0	0.0		40.0	16.6

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Ecological Archives E089-094-A4

Craig I. Peter and Steven D. Johnson. 2008. Mimics and magnets: The importance of color and ecological facilitation in floral deception. *Ecology* 89:1583–1595.

Appendix D. A figure showing the average reflectance spectra of *Eulophia zeyheriana* and *Wahlenbergia cuspidata* flower parts.

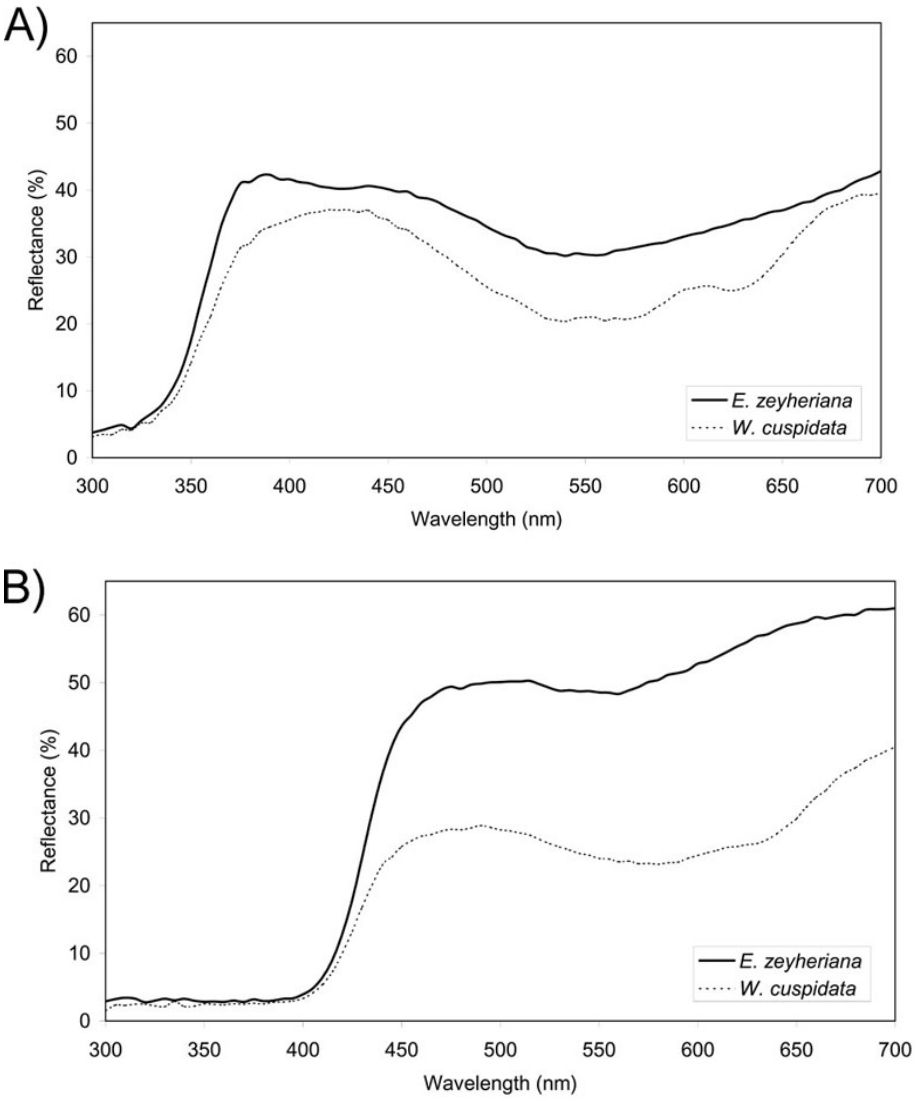

FIG. D1. Average reflectance spectra of *E. zeyheriana* and *W. cuspidata* (A) petals and (B) labellum/pollen presenter. $n = 22$ for each of the averages curves.

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Ecological Archives E089-094-A5

Craig I. Peter and Steven D. Johnson. 2008. Mimics and magnets: The importance of color and ecological facilitation in floral deception. *Ecology* 89:1583–1595.

Appendix E. A table showing the results of a breeding system experiment for *Eulophia zeyheriana*.

TABLE E1: Comparison of *E. zeyheriana* capsule and seed quality produced by self- and cross-pollination.

	Cross-pollinated $n = 16$	Self-pollinated $n = 17$	t_{31}	P
Fertile seeds (%)	80.07	52.36	3.91	<0.001
Capsule and seeds mass (g)	0.08	0.07	0.54	0.56
Seed mass (g)	0.01	0.01	0.15	0.88

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Cross-pollination mechanisms

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Anther Cap Retention Prevents Self-pollination by Elaterid Beetles in the South African Orchid *Eulophia foliosa*

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• *Background and Aims* Pollination by insects that spend long periods visiting many flowers on a plant may impose a higher risk of facilitated self-pollination. Orchids and asclepiads are particularly at risk as their pollen is packaged as pollinia and so can be deposited on self-stigmas en masse. Many orchids and asclepiads have adaptations to limit self-deposition of pollinia, including gradual reconfiguration of pollinaria following removal. Here an unusual mechanism—anther cap retention—that appears to prevent self-pollination in the South African orchid *Eulophia foliosa* is examined.
• *Methods* Visits to inflorescences in the field were observed and pollinators collected. Visitation rates to transplanted inflorescences were compared between a site where putative pollinators were abundant and a site where they were rare. Anther cap retention times were determined for removed pollinaria and atmospheric vapour pressure deficit was recorded concurrently. Anther cap anatomy was examined using light microscopy.
• *Key Results Eulophia foliosa* is pollinated almost exclusively by *Cardiophorus obliquemaculatus* (Elateridae) beetles, which remain on the deceptive inflorescences for on average 301 s ($n = 18$). The anther cap that covers the pollinarium is retained for an average of 512 s ($n = 24$) after pollinarium removal by beetles. In all populations measured, anther cap dimensions are greater than those of the stigmatic cavity, thus precluding the deposition of self-pollinia until after the anther cap has dropped. An anatomical investigation of this mechanism suggests that differential water loss from regions of the anther cap results in opening of the anther cap flaps. This is supported by observations that as atmospheric vapour pressure deficits increased, the duration of anther cap retention was reduced.
• *Conclusions* Flowers of *E. foliosa* are specialized for pollination by elaterid beetles. Retention of anther caps for a period exceeding average visit times by beetles to inflorescences appears to prevent facilitated self-pollination in *E. foliosa* effectively.

Key words: *Eulophia foliosa*, Orchidaceae, Elateridae, pollinarium reconfiguration, pollinia, anther cap retention, geitonogamy, anti-selfing mechanism, beetle pollination.

INTRODUCTION

In both the Orchidaceae and the Asclepiadoideae (Apocynaceae), pollen is usually packaged in units known as pollinia. In both groups the pollinia are associated with a variety of accessory structures, making up a pollinarium—the functional unit removed from the flower by the pollinators (Bookman, 1981; Johnson and Edwards, 2000). In most monandrous orchids, only a single visit is required to remove the entire complement of pollen from a flower. If the species is self-compatible then self-pollination, either through geitonogamy or facilitated autogamy, may be particularly crippling as it potentially involves all of the pollen and ovules of a flower. Selfing affects the female function of flowers resulting in inbred progeny (Darwin, 1878; Charlesworth and Charlesworth, 1987) as well as ovule discounting (Barrett, 2002). Selfing can also affect the male function of flowers, through the loss of potential to export pollen to other plants, a process known as pollen discounting (Barrett, 2002).

Many orchids and asclepiads have adaptations to reduce the likelihood of self-pollination. In orchids the absence of a reward in many species may be an important mechanism to discourage repeat visits on an inflorescence (Dressler, 1981; Johnson and Nilsson, 1999; Johnson *et al*., 2004). A more obvious adaptation, first recognized in orchids by Darwin (1867), is the change in orientation that pollinaria undergo following removal. In both orchids and asclepiads, freshly removed pollinaria are orientated such that if the pollinator immediately revisited the flower, or other flowers on the inflorescence, the pollinia would be incorrectly orientated to be deposited on the stigma. It is only after the accessory structures of the pollinaria, such as the caudicles or stipes in orchids (Johnson and Edwards, 2000) and translator arms in asclepiads (Bookman, 1981), change their shape after a specific interval that the pollinia are correctly orientated to strike the stigma. Orchid examples include those cited in Johnson and Edwards (2000) and Peter and Johnson (2005), while Queller (1985) describes changes in pollinarium orientation in *Asclepias exaltata*.

Two other less commonly documented mechanisms associated with pollinaria to avoid self-depositions of pollinia have been recognized. Borba and Semir (1999) found that pollinia of two species of *Bulbophyllum* have to dry and shrink over about 2 h before they can be inserted into the stigmatic cavity. This mechanism has also been reported in *Trigonidium obtusum* (Singer, 2002).

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A second mechanism has been demonstrated in some epidendroid orchids and involves the retention of the anther cap – anther tissue that covers the pollinarium while it is still in place on the column. In most species, the anther cap immediately drops off the pollinarium following its removal by a pollinator, but in a few species the anther cap clasps the pollinia, making them unavailable for a period, before the anther cap drops off. This delay may function as a mechanism to reduce geitonogamous pollen deposition (Dressler, 1981).

van der Pijl and Dodson (1966) cite anther cap retention times of about 20 min in a number of species of *Catasetum* and 2–3 h in *Cycnoches lehmanni*. Singer and Cocucci (1999) documented anther cap retention times of up to 40 min in *Pleurothallis luteola*, and Borba and Semir (2001) recorded anther cap retention times of up to 30 min in *Pleurothallis teres* and *P. ochreata*. All three of these small South American orchids are pollinated by small flies (Phoridae and Chloropidae) which spend considerable time visiting individual inflorescences, apparently collecting rewards. Both studies suggest anther cap retention is a mechanism to limit geitonogamous pollen transfer by insects that spend considerable time visiting an individual plant.

Catling and Catling (1991) recorded anther cap retention times of between 8 and 110 min in *Tipularia discolor*, depending on the ambient relative humidity. At ambient relative humidity of 60–65 % anther caps were retained for between 30 and 40 min. At a relative humidity of 90–93 % this increased to about 100 min. The authors noted that the two flaps of the anther cap that envelope the pollinia gradually open until the anther cap no longer grasps the pollinia and so falls off. This observation coupled with the increased anther cap retention times at high relative humidity point to the changing water status of the cells of the anther cap as the primary mechanism behind the delayed dropping of the anther cap. The anther cap in *T. discolor* is small enough to fit into the stigmatic cavity. However, the differing microrelief of the pollinia compared with that of the anther cap effectively limits deposition in comparison with those pollinaria without anther caps.

Preliminary observations of the South African orchid *Eulophia foliosa* indicate that small click beetles (Elateridae) are the main visitors. Furthermore, the pollinaria were noted to have persistent anther caps. We hypothesized that anther cap retention in *E. foliosa* is a mechanism that functions to limit self-pollination in a plant specialized for pollination by slow-moving beetles.

Beetle pollination is under-studied, although Bernhardt (2000) reviewed specialized beetle pollination in 184 angiosperm species in 34 families. Beetle pollination is much less common in species with zygomorphic flowers. These include the orchid examples listed below, as well the remarkable *Orchidantha inouei* (Lowiaceae) pollinated by dung beetles (Sakai and Inoue, 1999). In South Africa, beetles may be important pollinators, with estimates of up to 34 % of plant taxa being beetle-pollinated in some ecosystems (Bernhardt, 2000). Monkey beetles (Hopliini, Rutelinae, Scarabaeidae) are important pollinators of many geophytes (Goldblatt *et al*., 1998). Flower chafer beetles (Cetoniinae, Scarabaeidae) are perhaps equally important in the eastern parts of South Africa (C. I. Peter, pers. obs.).

There are fewer than ten reports of beetle pollination in the Orchidaceae. Most involve systems in which beetles are part of mixed pollinator assemblages. For example, *Listera ovata* is visited by 283 species of insects but primarily Ichneumonid wasps, saw flies (Tenthredinidae) and beetles such as Elateridae, Cantharidae and Bruchidae (Nilsson, 1981). Another European orchid, *Coeloglossum viride*, is pollinated by both wasps and beetles. The latter include Elateridae and Cantharidae (Silén, 1906, cited in Proctor *et al*., 1996; C. I. Peter *et al*., unpubl. data). Gutowski (1990) noted cerambycid beetles pollinating the European orchid *Dactylorhiza fuschii* in the forests of north-eastern Poland, but elsewhere in Europe this species is pollinated by bumble bees and honeybees (Dafni and Woodell, 1986).

Examples of more specialized beetle pollination systems in orchids are starting to emerge from the southern hemisphere. These include the unusual *Pterglossaspis ruwenzoriensis*, which is found both in central Africa and in South America. The flowers are dark purple inside and arranged on relatively crowded inflorescences. The yeasty scent is probably produced from the papilose adaxial surface of the labellum. A 'jelly-like' nectar is secreted from the base of the column. In Argentina, this species is pollinated by the cetonid beetle *Euphora lurida* (Singer and Cocucci, 1997).

Steiner (1998*a*) reported that the deceptive South African orchid *Ceratandra grandiflora* is specialized for pollination by monkey beetles (Hopliini, Scarabaeidae). Unlike those of related species, the yellow flowers of *C. grandiflora* are crowded into capitate inflorescences, providing a large landing platform for the beetles. Steiner interpreted this as a case of generalized food deception coupled with rendezvous pollination, as beetles were often observed mating in the flowers.

The aims of this study therefore were to establish whether (1) anther cap retention can function to reduce pollinator-mediated self-pollination in *Eulophia foliosa* and (2) a specialized beetle pollination system occurs in this species.

MATERIALS AND METHODS

Study species

Eulophia foliosa (Lindl.) Bolus is a terrestrial orchid about 30 cm in height. The inflorescence emerges concurrently with the vegetative shoot from its base (Fig. 1A). The inflorescence bears numerous small flowers in crowded inflorescences of up to 70 flowers (average 40, Fig. 1B). Each flower is pale green with a dark maroon labellum. Two solid pollinia forming a single pollinarium are housed at the end of a relatively long, slender column beneath an enclosing anther cap. The flowers provide no discernible reward, and are faintly honey-scented to humans. The vegetative shoot bears two or three erect plicate leaves (Fig. 1A). This species grows in open grassland throughout much of the eastern part of South Africa from near sea-level to approximately 2000 m in the Drakensberg Mountains, but

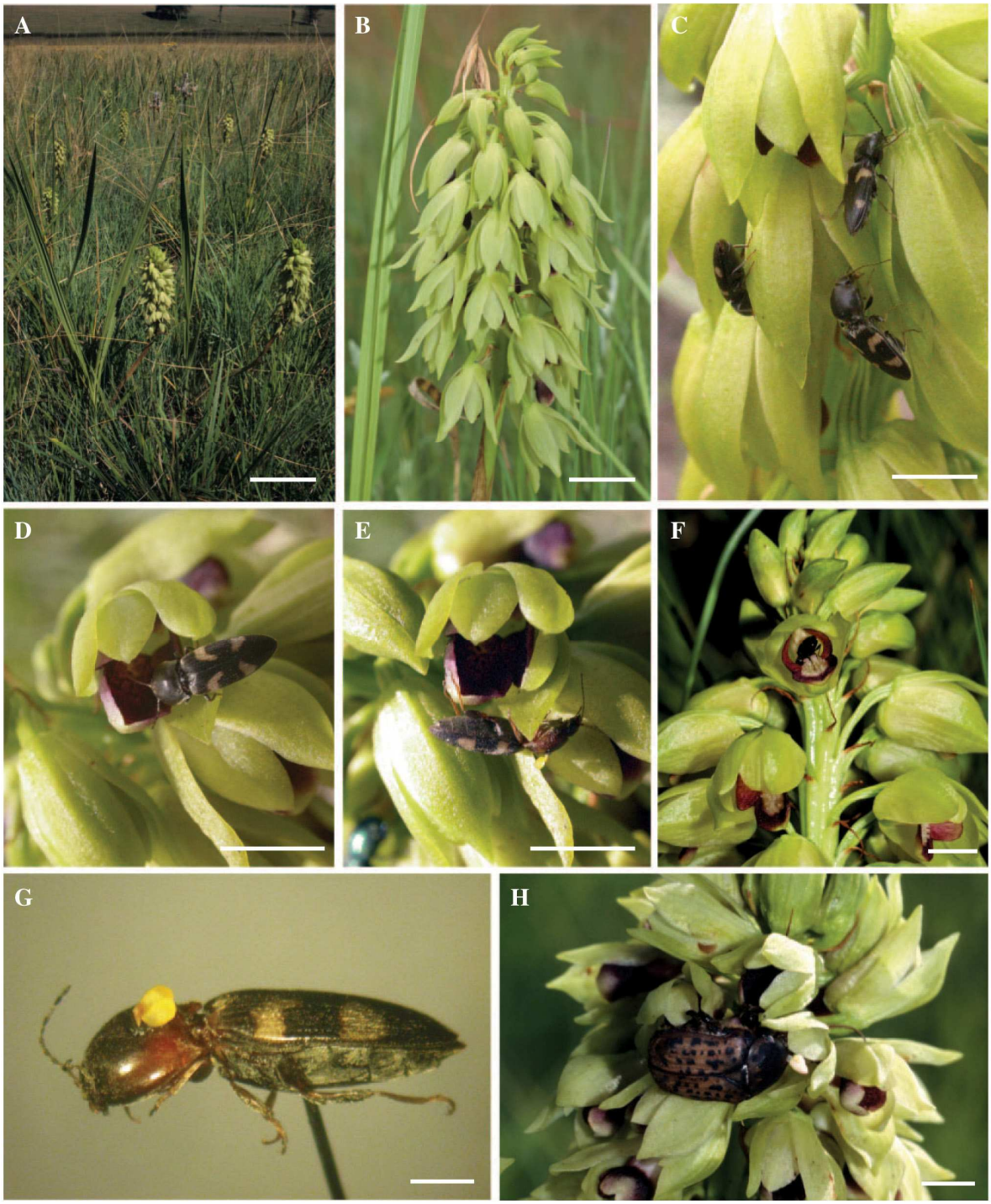

FIG. 1. *Eulophia foliosa* occurs in large populations in grasslands throughout the eastern parts of South Africa (A). The inflorescences are crowded with many apple-green flowers (B). In most populations inspected, numerous *Cardiophorus obliquemaculatus* (Elateridae) clickbeetles (G) were found visiting the inflorescences (C–F). Occasionally at higher altitude sites in KwaZulu-Natal we observed the much larger *Atricelaphinis tigrina* (Cetoniinae) visiting the inflorescences and removing the pollinaria (H). Scale bars = A, 100 mm; B, 20 mm; C–F, H, 5 mm; G, 1 mm.

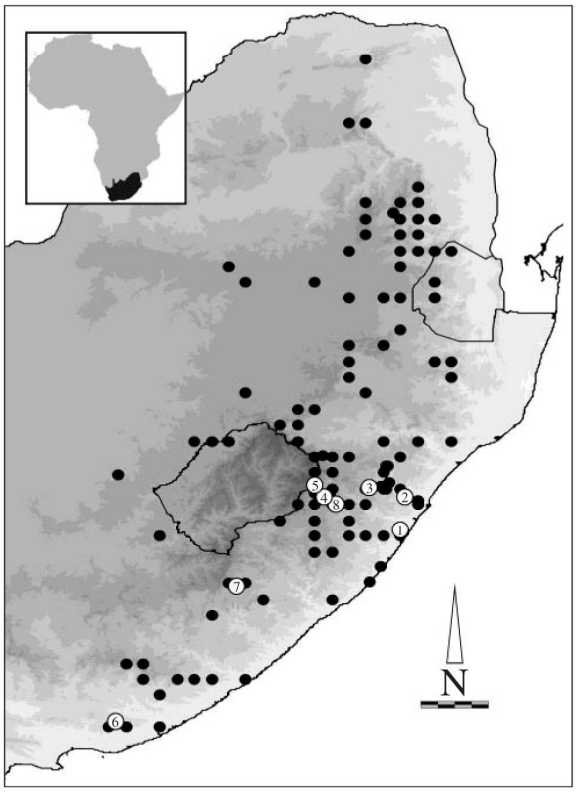

FIG. 2. Distribution of *Eulophia foliosa* in South Africa. Study sites include (1) Vernon Crookes Nature Reserve, (2) Camperdown, (3) Wahroonga, (4) Himeville Nature Reserve, (5) Sani Pass (all in KwaZulu-Natal) and (6) Dassie Kranz near Grahamstown in the Eastern Cape Province. Additional observations of *Cardiophorus obliquemaculatus* visiting *E. foliosa* inflorescences were made at (7) Ugie as well as Pevensy and a site east of Underberg (both indicated by point 8). Increasing altitudes are shown in darker shades of grey. Scale bar = 100 km.

is absent above this altitude in the Drakensberg. The distribution extends from Grahamstown in the south northwards through eastern South Africa to Limpopo Province in the far north of South Africa (Fig. 2) and parts of Zimbabwe (Thomas, 1998).

Study sites

Field work was undertaken at a number of sites in the KwaZulu-Natal and Eastern Cape provinces of South Africa, ranging from around 300 m near the coast to approx. 2000 m in the Drakensberg Mountains. Sites in KwaZulu-Natal include Vernon Crookes Nature Reserve, Camperdown, Wahroonga, Himeville Nature Reserve and Sani Pass below the South African border post (Fig. 2). In the Eastern Cape Province a large population was investigated at Dassie Kranz on the summit of the Rietberg south of Grahamstown (Fig. 2). Additional observations of visits to the inflorescences of *E. foliosa* were made near Pevensy, east of Underberg (KwaZulu-Natal) and near Ugie (Eastern Cape).

Pollinator observations

Insects were observed visiting the inflorescences at most of the study sites and, at a number of the sites, insects were collected bearing pollinaria (Table 1). Insects were collected directly into an ethyl acetate killing jar or first knocked into an insect net beside the inflorescence before being killed. Identified insects are lodged in the collection of the first author and the Albany Museum, Grahamstown. Visits to the inflorescences were observed at a number of sites, and in the Grahamstown population it was possible to time the duration of these visits. Behaviour of beetles on inflorescences was recorded using a digital dictaphone. The duration of the visits in seconds was determined from software used to play the recordings. Pollinators bearing pollinaria of *E. foliosa* were also collected visiting flowers of other species, primarily *Helichrysum nudifolium* (Asteraceae), in the vicinity of the orchids.

Transplant experiment

In 2001 and 2002, despite large numbers of *E. foliosa* plants in flower in Himeville Nature Reserve, pollinaria removal and deposition rates were observed to be nearly zero. Intensive searches failed to find any elaterid beetles in the area. At the nearby Sani Pass site, within the range of *E. foliosa* but without a natural population of this orchid in the vicinity, elaterid beetles were numerous. To determine whether the lack of pollination at the Himeville site was a simple consequence of the lack of beetles, 60 inflorescences of *E. foliosa* were collected and 30 of these were 'transplanted' to Himeville Nature Reserve (pollinators rare) and 30 to the Sani Pass site (pollinators present). Inflorescences were first inspected and flowers showing signs of visitation were removed. They were then positioned at a natural height above the grass canopy with the cut ends of the scape in vials of water.

After 3 d the transplanted inflorescences were collected and pollinaria removal and deposition were determined. Data for the two sites were compared using the Mann–Whitney *U*-test. From these data, pollen transfer efficiency (PTE), the percentage of removed pollinia that are deposited on stigmas (cf. Johnson *et al*., 2004), was determined. PTE for a number of other populations, including Grahamstown, Ugie and Pevensy, was also determined.

Breeding systems

The breeding system of this species was investigated in the Grahamstown population. Pollinators were excluded from the inflorescences using black netting bags and flowers were either selfed or pollinated with pollinia from plants growing more than 20 m away. In addition, flowers were marked and left unmanipulated to test for autogamy, which is common in *Eulophia* (Williamson, 1984; C. I. Peter, unpubl. data). Each inflorescence had only one self-pollinated and one out-crossed flower, thus avoiding pseudoreplication. More flowers were marked as unmanipulated controls but these were also spread across many inflorescences. Seven unpredated inflorescences and

TABLE 1. *Pollinators and visitors to* Eulophia foliosa *observed at the various study sites*

Site number*	Site	Time (h)†	Visitors	Number	Number with pollinaria (%)
1	Vernon Crookes	7	*Cardiophorus obliquemaculatus*	6	3 (50)
			Elateridae	1	0
			Pompilidae	1	0
			Hopliniiae	2	0
			Unidentified Coleoptera	2	0
2	Drummond	9	*C. obliquemaculatus*	43	16 (36)
			Cardiotarsus species	9	0
			Cyrtothyrea marginalis	1	0
			Curculionidae	2	0
3	Wahroonga	4	*C. obliquemaculatus*	5	0
			Atricelaphinis tigrina	2	2 (100)
			C. marginalis	1	0
			Hopliniiae	6	0
4	Himeville	8	*A. tigrina*	5	2 (40)
5	Sani Pass	4	*C. obliquemaculatus*‡	approx. 5	0
6	Grahamstown	15	*C. obliquemaculatus*	32	7 (22)
			Pompilidae	1	0
7	Ugie	<1	*C. obliquemaculatus*	approx. 5	0
8	Pevensy	<1	*C. obliquemaculatus*	3	0
8	East of Underberg	<1	*C. obliquemaculatus*	3	0

* Corresponds with the site numbers given in Fig. 1.
† Estimated time spent observing the inflorescences at each site.
‡ On transplanted inflorescences.

five mature capsules were recovered for each of the self- and cross-pollinated treatments. A *t*-test was used to examine the differences between the quality of self-pollinated and cross-pollinated capsules and seeds set.

Pollinarium bending and anther cap retention

Pollinarium reconfiguration through bending is common in *Eulophia*. While attempting to record the bending rates of the pollinaria of *E. foliosa* it was observed that the anther caps were difficult to dislodge. In some cases forcefully removing the anther cap breaks the pollinia, still enclosed in the anther cap, from the stipe. The time it took for the anther cap to become loose enough to be removed by a moderate air current was therefore recorded.

Pollinaria were removed from the flower using the tip of a fingernail. Pollinaria were then held in an exposed position where they received sunlight and were exposed to approx. 15 km h^{-1} breeze (approx. 0·4 m s^{-1}). Suspecting this response to be driven by changing water status of the cells of the anther cap, we recorded ambient temperature and relative humidity using automatic data loggers. From these two measurements, atmospheric vapour pressure deficit (VPD) was calculated according to the equations of Goff and Gratch (1946). VPD describes the gradient of water from the cells to the atmosphere and assumes the cells of the anther cap to be wet and freely losing water. VPD is a more relevant measurement of the gradient of water from the plant to the environment than relative humidity alone (Monteith, 1965) as plants are known to respond directly to VPD (Lösch and Tenhunen, 1981).

Positioning of the pollinaria in a constant airflow is therefore important, not only as it provides constant agitation so that an endpoint (dropping of the anther cap) can be determined, but the breeze also reduces any boundary layer conditions around the pollinaria. These times were recorded for pollinaria from fresh flowers selected from random plants in the Grahamstown population from 0800 h until midday on 19 December, 2004. In most cases only one flower was used per inflorescence. For four inflorescences, anther cap retention times were recorded for two flowers.

Anther cap anatomy

Flowers were preserved in 70 % ethanol and dehydrated in an alcohol–butanol series before being embedded in paraplast wax. Sections of 10–15 µm thickness were cut using a microtome. Mounted sections were stained with safranin and fast green and then imaged.

Anther cap and stigma dimensions

The widths and lengths of anther caps from different populations were recorded with digital callipers, as were the widths and lengths of stigmas and the width of the two pollinia from the flower. Only one flower was measured per inflorescence.

RESULTS

Pollinators

Many visitors to the inflorescences were observed at all the sites investigated (Table 1). However, only two beetle species bore *E. foliosa* pollinaria. By far the most numerous visitors to the inflorescences were *Cardiophorus obliquemaculatus* (Elateridae) and at Vernon Crookes, Drummond and Grahamstown a number of individuals bearing pollinaria were collected (Table 1). At these sites, *C. obliquemaculatus* were observed to enter flowers and remove pollinaria. The beetles clamber all over the

inflorescence and probe the flowers wherever there is a space or depression such as those between petals. Where these attempts find the main opening of the flower, the curvature of the labellum forces the insect to assume the 'correct' orientation to enter the flower fully. This curvature also forces the elytra or thorax of the beetles up against viscidium of the pollinarium (Fig. 1F). The attachment of the anther cap–pollinarium unit to the column is firm, as is the viscidium attachment to the elytra or thorax of *C. obliquemaculatus*. As a result, the beetle may struggle for a number of minutes to remove the pollinarium from the column before it can exit the flower.

One beetle was also observed depositing pollinia on a stigma, a process that took in excess of 10 min and also entailed much struggling by the beetle. This observation is supported by a smaller number of beetles bearing visicidia, which suggests that these beetles may have deposited pollinia on stigmas.

Numerous visits of *C. obliquemaculatus* to inflorescences of *E. foliosa* were observed at the Grahamstown site. These visits ranged between 45 and 550 s (average 302 s) in duration and usually entailed the beetle probing only a few flowers per inflorescence—typically fewer than five. More than one *C. obliquemaculatus* beetle on an inflorescence was routinely observed, but beetles were never observed mating.

Beetles removing pollinaria were observed on five occasions at Grahamstown, twice at Drummond and once at Vernon Crookes. In all cases the beetles left the inflorescences with the anther caps still covering the pollinia and, although a few of these beetles probed other flowers following the removal of the pollinia, no beetles depositing pollinia on self-stigmas were observed.

Although a number of *C. obliquemaculatus* were also observed on the inflorescences at other sites (Wahroonga, Pevensy, Underberg and Ugie), no individuals bearing pollinaria were collected. This may be an artefact of limited observation time. At these sites (except Wahroonga) observations were limited to less than 1 h each (Table 1).

At Wahroonga and Himeville a few much larger *Atricelaphinis tigrina* (Cetoniinae) beetles visiting the inflorescences were collected. Some of these beetles removed pollinaria (Fig. 1H). No beetles were observed depositing pollinia and no beetles bearing viscidia, indicating that they may have deposited pollinia, were collected.

In addition, a number of other beetle visitors to the inflorescences were observed, including larger elaterids (*Cardiotarsus* species), weevils (Curculionidae) and occasionally monkey beetles (Hopliini, Scarabaeidae). A few visits to the inflorescence by two small pompilid wasps were observed (Grahamstown, Vernon Crookes) and a scoliid wasp (Hesketh Conservation Area, Pietermaritzburg, South Africa). None of these insects bore pollinaria of *E. foliosa*.

Transplant experiment

The mean proportion of flowers with pollinaria removed from transplanted inflorescences was significantly higher at Sani Pass, where *C. obliquemaculatus* were common, compared with Himeville Nature Reserve, where pollinators were rare ($Z = 2{\cdot}44$, $P = 0{\cdot}015$). This was despite the activity of a troop of baboons that disturbed many inflorescences apparently chewing on many of them, removing flowers and substantially reducing our sample size (Table 2). The mean proportion of flowers with pollinia deposited was low at Sani Pass (0·004) and zero at Himeville. This difference was not significant ($Z = 1{\cdot}12$, $P = 0{\cdot}264$).

PTE was nil at Himeville, where none of the eight pollinia that were removed (four pollinaria from 951 flowers) were deposited on stigmas in the transplanted group. PTE in the plants transplanted to the site where pollinators were abundant was considerably higher. Three of the 38 removed pollinia (19 pollinaria) were deposited on stigmas (7·9 %), which is within the range of PTE recorded in natural populations (Table 2).

PTE at other sites ranged from 6·8 % at Ugie to 20·7 and 26·9 % for the Grahamstown and Pevensy populations, respectively (Table 2). At the Grahamstown site, 9 % of flowers on inflorescences produced fruit ($n = 27$ plants).

TABLE 2. *Pollen transfer efficiency (PTE; cf. Johnson* et al.*, 2004) for both transplanted plants and natural populations*

Site	No. of flowers	Flowers with pollinaria removed (%)	Flowers with pollinaria deposited (%)	PTE (%)
Transplanted to Sani Pass (pollinators)	618	2.7	0.4	8
Transplanted to Himeville (pollinators rare)	951	0.4	0	0
Grahamstown	83	49	20	21
Ugie	60	48	7	7
Pevensy	37	35	19	27
Cobham	37	59	16	14

TABLE 3. *Breeding system of* Eulophia foliosa *(see text for statistical analysis)*

	Crossed	Selfed	Unmanipulated
Fruit set, % (*n*)	71 (7)	71 (7)	0 (37)
Mean capsule weight, grams (s.e.)	0.106 (0.033)	0.053 (0.015)	0
Mean seed weight, grams (s.e.)	0.020 (0.009)	0.004 (0.002)	0
Seeds with embryos, % (s.e.)	71.5 (10.0)	14.6 (6.63)	0

Breeding system

While fruit set was the same for both cross-pollinated and self-pollinated treatments, the quality of self-pollinated fruit in terms of capsule weight and seed weight was lower (Table 3), although this was not significant ($t_{12} = 1{\cdot}26$, $P = 0{\cdot}23$ and $t_{12} = 1{\cdot}59$, $P = 0{\cdot}14$, respectively). However, cross-pollinated capsules produced significantly more fertile seeds than self-pollinated seeds ($t_8 = 4{\cdot}57$, $P = 0{\cdot}002$).

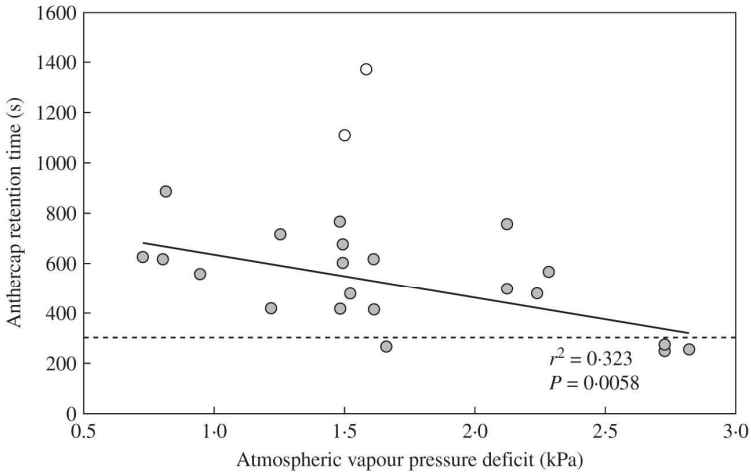

FIG. 3. Anther cap retention time decreases with increasing vapour pressure deficit (VPD), which describes the gradient of water vapour from the cells to the atmosphere. VPD is calculated from ambient temperature and relative humidity. Pollinaria were orientated in a moderate breeze of approx. 0·4 m s^{-1} to reduce boundary layer conditions around the pollinarium. Two outliers are from one specific plant and are excluded from the regression ($n = 21$). Dotted line indicates the average visit times of *Cardiophorus obliquemaculatus* to inflorescences.

There is no evidence of autogamy in *Eulophia foliosa* and pollinators are required for fruit set (Table 3).

Anther cap retention

Anther cap retention times ranged from 249 s during warm, dry conditions to 887 s in the cool, humid morning, with an average retention time of 512 s. This is significantly longer than the average time of 302 s the beetles spend on the inflorescences ($t_{43} = 11{\cdot}8$, $P = 0{\cdot}000$). This excludes the very long retention times in excess of 1000 s recorded for anther caps from a single plant in the population. This plant was also excluded from the regression in Fig. 3. The opening of the 'proximal flaps' of anther caps (arrowheads, Fig. 4) frees the anther cap from the rest of the pollinarium (Fig. 4). Once the anther cap has dropped, the exposed pollinia (Fig. 4D) are available for deposition on a subsequent visit. It is clear from Fig. 3 that this process occurs more rapidly when the water vapour gradient from the anther cap cell to the atmosphere is large during conditions of high VPD. This suggests that water loss is the primary mechanism behind dropping of the anther cap.

Interestingly, the pollinaria also undergo a change in orientation. This occurs in many other species of *Eulophia* and typically involves the stipe of the pollinarium bending through up to 180° so that the pollinia are orientated in the opposite direction to that when they are first removed. In this position, the pollinia can be scraped off by the distal lip of the stigma, bringing about deposition (C. I. Peter, unpubl. data). In *E. foliosa* bending takes approximately 60 s but this is difficult to determine as the movement of the pollinarium is masked by the anther cap, which envelopes the pollinia. Once the anther cap has dropped the pollinia are already correctly orientated to ensure that they strike the stigma.

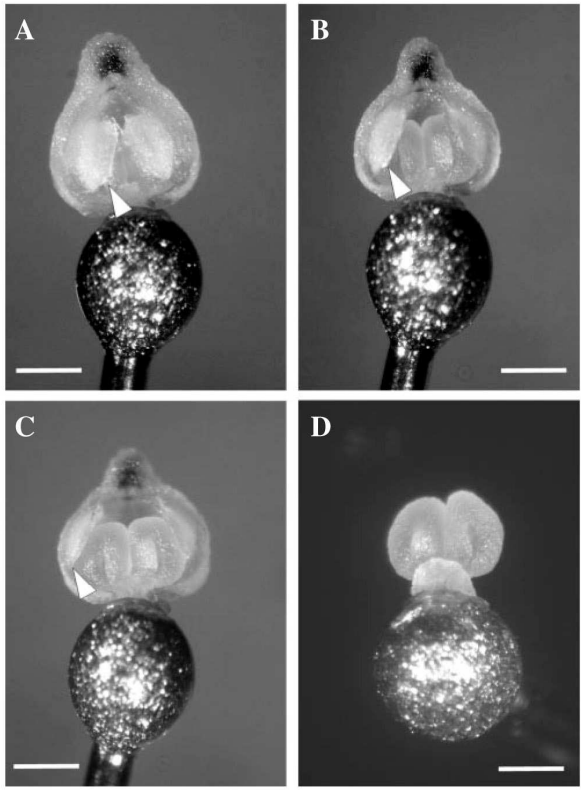

FIG. 4. View of the proximal (rostellum side) of a pollinarium removed from the flower on the head of an insect pin. (A) Pollinarium with anther cap in place—'flaps' of the anther cap (arrowheads) enclose the pollinarium in the anther cap. (B) Anther cap 'flaps' begin to open, (C) anther cap 'flaps' completely open and (D) anther cap blown off 9 min after removal, exposing the pollinia for deposition. Scale bar = 0·5 mm.

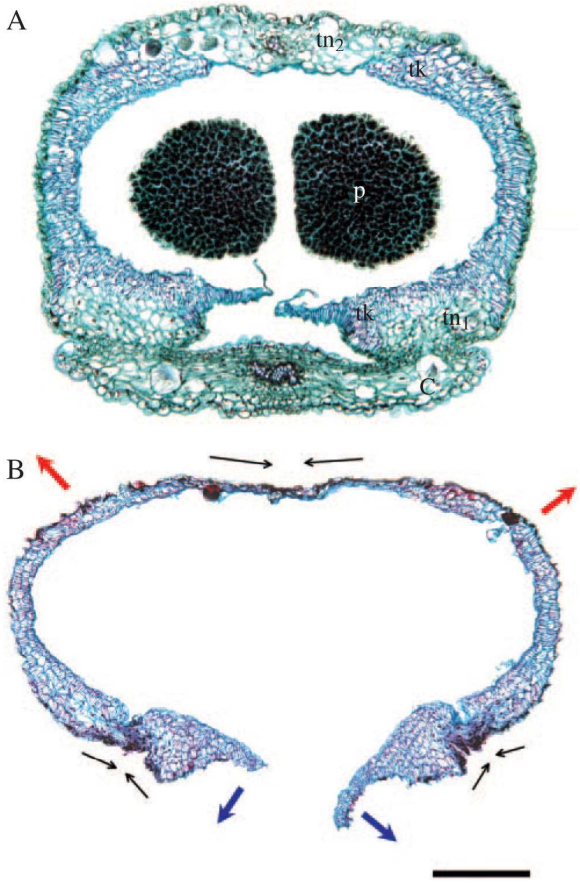

FIG. 5. (A) Anatomy of *Eulophia foliosa* anther cap and pollinia (p) in cross-section while still attached to the column (c). The anther cap is made up of both thick-walled (tk) and two zones of thin-walled (tn) cells. One zone of thin-walled, lightly stained cells marks the abscission layer of the anther cap from the column (tn_1); the second zone is a band of cells along the distal margin of the anther cap relative to the rest of the column (tn_2). (B) Cross-section of an anther cap dropped from the pollinarium following its natural opening sequence. Arrows are as explained in the text. Scale bar = 250 µm.

Anther cap anatomy

The anther cap is made up of four or five layers of cells in most places. Two regions of cells appear to play a role in the opening of the anther cap to expose the pollinia. There is a region of amorphous cells with thin cell walls, lightly stained with fast green. These cells mark the abscission layer where the anther cap attaches to the column (tn_1 in Fig. 5A). Adaxial to these cells are layers of smaller cells with thicker cell walls stained by both safranin and fast green, appearing purple (tk). Following anther cap removal, both the small thick-walled cells and large thin-wall cells appear dehydrated (Fig. 5B). However, the small thick-walled cells change dimensions only slightly relative to the large thin-walled cells, which shrivel substantially. As a result of this differential shrinking (Fig. 5B, fine arrows), the anther cap flaps open outwards (Fig. 5B, blue arrows).

A second mechanism also involves the differential shrinking of the cells along a band marking the distal edge of the anther cap relative to the column (tn_2 in Fig. 5A). There is a single layer of thick-walled cells forming the adaxial layer of cells of the anther cap. Abaxial to this are poorly defined thin-walled cells (tn_2), which appear to shrink and contract relative to the single layer of thick-walled cells (in the direction of the fine arrows, Fig. 5B). This shrinking increases the distance between the two flaps (Fig. 5B, red arrows), further opening the anther cap to facilitate its dropping. This flaring is evident when comparing the width of the anther cap in Fig. 4A and B.

Anther cap and stigma dimensions

Anther cap widths are consistently wider than the stigmas in all populations (Table 4), although individual flowers occasionally had stigmas wider than their anther cap. Averaged across all populations, the anther caps were 0·13 mm (11 %) wider than the stigmas. There was little variation in these measurements within flowers found on a single inflorescence (data not shown). The stigmas were on average 0·31 mm (25 %) wider than the paired pollinia (Table 4). Besides these measurements, the lengths of the anther caps were also measured. The anther caps were substantially longer than the stigmas, the average for all populations being 0·86 mm.

Over the course of the study, the stigmas of 1869 flowers were inspected as part of the PTE calculations. In only one instance was an anther cap found clogging a stigma.

DISCUSSION

The results of this study are consistent with the idea that anther cap retention in *Eulophia foliosa* serves a functional role to prevent facilitated self-pollination by the slow-moving beetles that are the primary pollinators of this species. In this species, pollinaria that retain their anther caps after removal are too large to be inserted into the stigmatic cavities of the flowers. This mechanism thus differs from that reported for *Tipularia discolor*, in which the anther cap is small enough to be inserted into the stigma, but prevents physical adherence to the stigma surface (Catling and Catling, 1991).

The possession of a mechanism to prevent facilitated self-pollination may be of particular benefit to 'epidendroid' orchids, as these orchids have only a few solid pollinia per flower (two in this case), which often function as a single unit. Self-insertion of these pollinia could compromise fitness because of both inbreeding depression in selfed progeny, loss of ovules that would otherwise have been cross-fertilized, and pollen discounting, the loss of pollen that would otherwise have been exported as part of the male function of a plant (Barrett, 2002). Self-pollination may be less detrimental for orchids with 'orchidoid'-type pollinaria, which are massulate, having pollen packed into smaller subunits (Johnson and Edwards, 2000). Even if some of these massulae are wasted on self-stigmas, there are probably

TABLE 4. *Comparison of anther cap and pollinia widths with the widths of stigmas in different populations of* Eulophia foliosa

Population	Anther cap width (mm)	Stigma width (mm)	Pollinia width (mm)	Anther cap wider than stigma by (mm)	Stigma wider than pollinia by (mm)	*n*
Grahamstown	1.24 (0.06) a	1.09 (0.06)	0.70 (0.03) a	0.15 (0.10)	0.39 (0.07)	5
Himeville	1.47 (0.03) b	1.30 (0.03)	1.04 (0.04) b	0.17 (0.04)	0.26 (0.02)	5
Drummond	1.30 (0.10) ab	1.12 (0.07)	0.87 (0.03) ab	0.19 (0.04)	0.25 (0.04)	2
Pevensy	1.36 (0.04) ab	1.19 (0.07)	0.91 (0.08) ab	0.18 (0.06)	0.28 (0.12)	4
Ugie	1.26 (0.04) a	1.22 (0.09)	0.93 (0.09) ab	0.04 (0.05)	0.30 (0.05)	5
Average	1.33	1.19	0.89	0.14	0.30	*n* . . . *n* = 21

Means that are not significantly different (Tukey test) share common letters. Standard error is given in parentheses.

many more massulae per pollinarium available for later export.

Although polliniarium reconfiguration through bending is by far the most commonly documented mechanism that prevents self-pollination in orchids and asclepiads (cf. Peter and Johnson, 2005), there are a number of reports of anther cap retention (this study; van der Pijl and Dodson, 1966; Dressler, 1981; Catling and Catling, 1991; Singer and Cocucci, 1999; Borba and Semir, 2001) as well as pollinia shrinking (Borba and Semir, 1999; Singer, 2002). Peter and Johnson (2005) compared reconfiguration mechanisms in orchids and asclepiads. They found that in species with pollinaria that reconfigure through a bending mechanism, these movements are rapid, taking less than 150 s on average. In all reported cases of anther cap retention and pollinia shrinking, reconfiguration times are considerably longer (this study; van der Pijl and Dodson, 1966; Catling and Catling, 1991; Singer and Cocucci, 1999; Borba and Semir, 2001). This suggests there is an upper time limit after which pollinarium bending can no longer precisely protect against selfing. Bending mechanisms probably entail differential water loss from small regions of tissue at the base of the stipe (Darwin, 1867) or from regions of the stipe itself (our pers. obs.). As a result, controlled water loss from small regions of tissue is only possible over the lower range of reconfiguration times. Where visit times are longer, slower pollinarium reconfigurations are only possible through alternative mechanisms that entail water loss from larger regions of tissue such as whole pollinia or large parts of the anther cap.

Little is known about the once-off reconfiguration movements of pollinaria. Darwin (1867) attributed the movement of pollinaria to the loss of water from regions of the viscidium or stipe. He also showed that in some cases the pollinarium bent back to its initial position when placed in water. The movement of pollinaria and anther cap flaps described here is distinct from nastic movements of leaves and flowers that repeatedly move (van Doorn and van Meeteren, 2003; Peter *et al*., 2004). In pollinaria and this example of anther cap retention, the movement is delayed and then proceeds to completion, reconfiguring the pollinia for deposition. The data presented here, both the correlation of anther cap retention times to atmospheric vapour pressure deficit and the observation of anther cap anatomy, point to water loss from specific regions of cells as the mechanism behind the opening of the anther cap to expose the pollinia. However, little is known about the anatomy of orchid anthers, and further study is needed (R. L. Dressler, pers. comm.).

Both anther cap retention and pollinium shrinking reconfiguration mechanisms are likely to be restricted to the 'higher epidendroid' orchids (*sensu* Cameron *et al*., 1999), with solid pollinia covered by anther caps. Species known to retain their anther caps are scattered through at least four tribes (*sensu* Cameron *et al*., 1999) in the sub-family and there is no obvious phylogenetic pattern to the occurrence of this phenomenon. It is likely that anther cap retention occurs in many more species of epidendroid orchids, particularly species with small, rewarding flowers pollinated by slow-moving insects which spend long periods on an inflorescence.

This mechanism of anther cap retention is different to that of all other *Eulophia* species we have investigated, where the anther cap drops off the removed pollinarium almost immediately. Anther cap retention in *E. foliosa* is probably a derived condition which protects this species against self-pollination by slow-moving elaterid beetles which spend considerable periods on its inflorescences. This is supported by our observation of beetles removing pollinaria over the course of long visits to inflorescences, but in all cases anther caps prevented self-deposition and promoted pollinia export. Actual rates of self-pollination in *E. foliosa* are difficult to measure as the pollinia cannot be labelled using stains (Peakall, 1989) or tags (Nilsson *et al*., 1992) without disturbing the anther cap function.

Beetle pollination appears to be fairly common in the South African flora, but most documented cases involve brightly coloured and unscented flowers that are pollinated by Hopliinid monkey beetles (Goldblatt *et al*., 1998; Steiner, 1998*b*; Bernhardt, 2000; Johnson and Midgley, 2001). The cryptic dull green scented flowers of *E. foliosa* (Fig. 1) are not consistent with this pollination system, instead showing similarities to flowers of orchids for which other beetles have been shown play a role in pollination (Nilsson, 1981; Singer and Cocucci, 1997). These include *Listera ovata* and *Coeloglossum viride* in the northern hemisphere, both of which, interestingly, are also visited by elaterid beetles (Nilsson, 1981; Silén 1906, cited in Proctor *et al*., 1996; C. I. Peter *et al*., unpubl. data).

Eulophia foliosa has non-rewarding flowers, unlike many other beetle-pollinated orchids, such as *L. ovata*, *C. viride* and *Pteroglossaspsis ruwenzoriensis.* This is in line with other species of *Eulophia*—of approx. 30 other species examined to date, all are deceptive and offer no reward

(C. I. Peter, unpubl. data). Steiner (1998*a*) suggested a system of rendezvous pollination for *Ceratandra grandiflora*, the only other deceptive orchid known to be beetle pollinated. None of the many *C. obliquemaculatus* beetles observed visiting the inflorescences of *E. foliosa* were mating, so the use of flowers as a rendezvous site is unlikely to be important in this system.

Specialized pollination by the elaterid beetle *C. obliquemaculatus* seems likely in *E. foliosa* given the fact that this insect is the sole pollinator at many sites across the geographical range of the species (Fig. 2), and the transplant experiment also shows an association between pollination success and the presence of these beetles (Table 2). This is a third example of specialized beetle pollination as reported in two other species of southern hemisphere orchids (Singer and Cocucci, 1997; Steiner, 1998*a*).

The pollination biology of the large, primarily African genus *Eulophia* remains largely undocumented, but it is probable that specialization for beetle pollination may be found to occur in other *Eulophia* species with flowers that have shallow or absent spurs (C. I. Peter, unpubl. data).

CONCLUSIONS

The data presented here suggest that anther cap retention for a period that exceeds the average visit times by pollinators prevents geitonogamous pollen deposition in *Eulophia foliosa*, an orchid pollinated exclusively by the elaterid beetle *Cardiophorus obliquemaculatus*. This delayed movement is in response to the differential shrinkage of tissue of the anther cap driven by the loss of water from these cells. As such, it represents a rare example of an orchid specialized for pollination by beetles.

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Doing the twist: a test of Darwin's cross-pollination hypothesis for pollinarium reconfiguration

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Mating success in plants depends largely on the efficiency of pollen dispersal. For hermaphrodite plants, self-pollination, either within or among flowers, can reduce mating opportunities because of pollen and ovule discounting and inbreeding depression. Self-pollination may be particularly detrimental in plants such as orchids and asclepiads that package each flower's pollen into one or more pollinia which, together with accessory structures, comprise a pollinarium. Darwin proposed that physical reconfiguration of pollinaria serves as a mechanism for reducing the likelihood of self-pollination. To be effective, the time taken for pollinarium reconfiguration would need to exceed that spent by a pollinator on a plant. We investigated pollinarium reconfiguration (including pollinarium bending, pollinium shrinking and anther cap retention) in 19 species and found a strong positive relationship between reconfiguration time and the duration of pollinator visits. Reconfiguration times were also consistently longer than pollinator visit times. These results provide strong support for Darwin's idea that this mechanism promotes cross-pollination.

Keywords: self-pollination; out-crossing; pollinia; pollination

1. INTRODUCTION

> …the movement of depression in the pollinia does not commence (as I know by trial) until the pollinia are fairly withdrawn out of their cells [anthers]; nor will the movement be completed, and the pollinia be fitted to strike the stigmatic surfaces, until about half a minute has elapsed, which will give ample time for the moth to fly to another plant, and thus effect a union between two distinct individuals
> (Darwin 1862, p. 31, commenting on the common European orchid *Orchis* [*Anacamptis*] *pyramidalis*.)

Pollinator-mediated self-pollination can strongly depress fitness in plants. Female fitness is most obviously affected if it leads to inbreeding depression in progeny (Charlesworth & Charlesworth 1987; Darwin 1878; Keller & Waller 2002), but self-pollination can also reduce the pool of pollen available for export to other plants and can thus also reduce male fitness through pollen discounting (Barrett 2002*a*; Herlihy & Eckert 2002). Many plants, regardless of their degree of genetic self-incompatibility, possess physical mechanisms for promoting cross-pollination (Barrett 2002*b*). Well-documented mechanisms include dichogamy (differences in maturity of male and female organs; Bertin & Newman 1993), herkogamy (the spatial separation of male and female organs (Barrett 2002*b*), which includes stylar polymorphisms such as di- and tristyly (Cesaro & Thompson 2004; Barrett & Harder 2005)); 'flexistyly' (Li *et al.* 2001) and enantiomorphy (Jesson & Barrett 2002); rewardlessness (Dressler 1981; Johnson & Nilsson 1999; Johnson *et al.* 2004); and unisexuality (Barrett 2002*b*).

For orchids and asclepiads, which package their pollen into pollinia, self-pollination is potentially disastrous for three reasons. First, self-deposition of an entire pollinium may eliminate most or all of the opportunity for a flower's pollen to be exported (Johnson & Edwards 2000). Second, for self-compatible species, such as most orchids, ovules self-fertilized en masse are rendered unavailable for cross-fertilization (Barrett 2002*a*). Third, self-fertilization in orchids typically leads to rates of embryo abortion that are double those in seeds arising from cross-fertilization (Tremblay *et al.* 2005).

Pollinaria (comprising the pollen packets—the pollinia—as well associated accessory structures) often reorient gradually after withdrawal from the anther (figure 1*a*,*b*). This is typically due to bending or twisting of an accessory structure (such as a stipe or caudicle) that connects the pollinium to a sticky pad (the viscidium) in orchids (Johnson & Edwards 2000) or mechanical clamp (corpusculum) in asclepiads (Bookman 1981). These structures, in turn, attach the pollinium to the body of the pollinator. In orchids, the pollinium is rotated through an arc of 30–120° depending on the particular species. This movement is necessary for the pollinium to become orientated correctly for insertion into a stigma (figure 1*c*). In asclepiads the paired pollinia are initially flared at right angles, but reconfigure to be closely appressed to one another in the correct position to be inserted into the stigmatic chamber (Bookman 1981). Darwin was intrigued by this phenomenon and referred to it as a 'beautiful contrivance' that would function to reduce self-pollination if the time taken for its completion exceeds the duration of a pollinator's visit to a plant (Darwin 1862, p. 16). In the only previously published test of this idea, Johnson *et al.* (2004) confirmed that self-pollination in the European orchid *Anacamptis morio* does not take place unless pollinator visits exceed the time taken for pollinaria to undergo their bending movement. Other mechanisms of pollinarium reconfiguration may serve a similar function, including pollinia that shrink gradually to the correct size to be inserted into the stigmatic cavity (Borba & Semir 1999), and anther-caps that cover the pollinaria for a period following the pollinarium's removal (Catling & Catling 1991).

The timing of pollinarium reconfiguration varies extensively in orchids and asclepiads. If this duration is adaptive, then two logical predictions from Darwin's hypothesis are: (i) that variation in the

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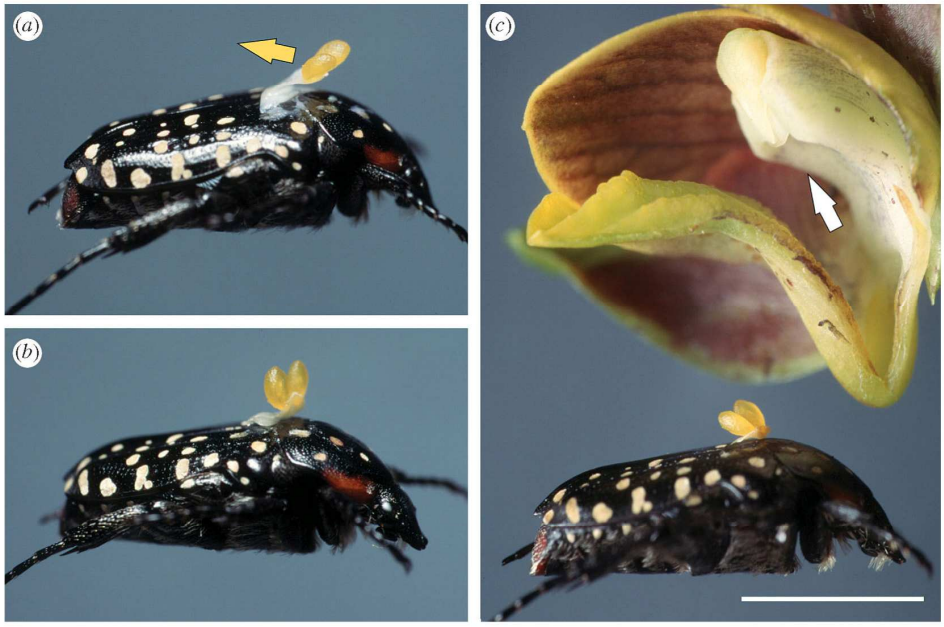

Figure 1. Pollinarium bending—the most common form of pollinarium reconfiguration. (*a*) A pollinarium of the orchid *Eulophia parviflora* freshly affixed to a cetoniid beetle will bend in the direction of the yellow arrow as indicated in (*b*) with the pollinarium half bent. (*c*) After *ca* 100 s the pollinarium has reconfigured and the paired pollinia can be inserted into the stigma (white arrow) as the beetle backs out of a flower in the direction of the white arrow (shown in cross-section). Scale bar, 5 mm.

Table 1. Characteristics and phylogenetic relationships of the study species.

taxon[a]	region[b]	type[c]	reconfiguration[d]	visit[d]	pollinator	reference
Acrolophia cochlearis	SA	B	88 (15)	28 (3)	bees	This study
Eulophia foliosa	SA	A	568 (26)	302 (18)	beetles	This study
Eulophia zeyheriana	SA	B	41 (13)	25 (2)	bees	This study
Eulophia parviflora (short spur)	SA	B	100 (32)	69 (5)	beetles	This study
Eulophia parviflora (long spur)	SA	B	28 (81)	24 (2)	bees	This study
Bulbophyllum involutum	Sm	S	7200 (15)	600 (*)	flies	(Borba & Semir 1999)
Bulbophyllum ipanemense	Sm	S	8100 (16)	600 (*)	flies	(Borba & Semir 1999)
Anacamptis morio	Eu	B	30 (20)	5 (12)	bees	(Johnson & Nilsson 1999)
Ophrys insectifera	Eu	B	307 (18)	310 (6)	wasps	(Kullenberg 1961)
Ophrys sphegodes	Eu	B	153 (7)	10 (c. 30)	bees	(Ayasse *et al.* 2000)
Neotinea ustulata	Eu	B	22 (15)	8 (2)	flies	This study
Orchis militaris	Eu	B	55 (19)	4 (4)	bees	This study
Orchis mascula	Eu	B	40 (20)	7 (26)	bees	(Johnson & Nilsson 1999)
Gymnadenia conopsea	Eu	B	28 (15)	7 (21)	moths	This study
Dactylorhiza viridis	Eu	B	874 (19)	514 (7)	generalist	This study
Platanthera chlorantha	Eu	B	80 (20)	11 (7)	moths	(Johnson & Nilsson 1999)
Platanthera blephariglottis	Nm	B	60 (*)	34 (58)	generalist	(Cole & Firmage 1984)
Asclepias exaltata	Nm	B	90 (*)	30 (*)	generalist	(Queller 1985)
Gomphocarpus physocarpa	SA	B	223 (20)	106 (21)	wasps	This study

[a]All taxa are orchids, except for *A. exaltata* and *G. physocarpa* which are asclepiads. Phylogenetic relationships among the taxa are indicated by the adjacent diagram.

[b]Region: SA, South Africa; Sm, South America; Eu, Europe; Nm, North America.

[c]Type of reconfiguration: B, bending; A, anthercap retention; S, pollinium shrinking.

[d]Mean time in seconds, sample size in parentheses, * sample size not reported.

timing of pollinarium reconfiguration reflects the duration of visits by a plant's pollinators, and (ii) that reconfiguration times of any particular species exceed the average duration of pollinator visits to plants of that species. Using data from 17 orchid and two asclepiad species, we explore the strength of support for Darwin's hypothesis that gradual reconfiguration of pollen after its removal from flowers serves as an

anti-selfing mechanism that is finely tuned to the duration of pollinator visits to plants.

2. METHODS

Data on the timing of pollinarium reconfiguration and pollinator visits to plants of 19 species were obtained from the literature (9 species) and our own observations in Sweden and South Africa (10 species; details and references given in table 1). Bending times of orchid pollinaria were recorded by withdrawing them from the anthers on the head of a pin and measuring their movement at suitable intervals with a protractor. Angles were plotted against time to determine when the pollinaria had stopped moving, which is unclear when pollinaria move slowly. The changing orientation of asclepiad pollinia was recorded by removing them from anthers with a hooked tip of an insect pin and photographing them at timed intervals using a dissecting microscope. Angles were measured from the subsequent photographs. In orchids in which pollinium insertion into stigmas is prevented by anther cap retention, the time taken for the anther cap to dry and fall off a pollinarium after it had been withdrawn was recorded. The duration of pollinator visits to inflorescences was recorded in the field with a portable voice recorder.

The relationship between observed values of pollinator visit time and pollinarium reconfiguration time was analysed using standard major axis regression (Legendre 2001), while the relationship between standardized linear phylogenetically independent contrasts (PICs) of these values was analyzed in the program PDTREE (Garland *et al.* 1992). The latter were based on a phylogeny of the 19 study species (table 1) obtained from existing phylogenetic trees (Cameron *et al.* 1999; Bateman *et al.* 2003) trimmed to include just the 19 species of interest. Branch lengths were assigned according to Pagel's arbitrary method (Pagel 1992). Relationships within *Eulophia* have not been fully resolved and are based on an existing classification (Hall 1965). Changing the position of the three *Eulophia* species in the phylogeny had no influence on the results using PICs. The variance homogeneity of contrasts was verified by examining the linear relationship between the absolute value of the standardized contrasts and the sum of the squares of branch lengths (Garland *et al.* 1992).

3. RESULTS

The average time taken for a species' pollinaria to reconfigure varied positively with the average time that pollinators spent visiting individual plants, both when actual values and phylogenetically independent contrasts are considered (figure 2*a*,*b*). Importantly, the duration of pollinarium reconfiguration almost always (in 18/19 cases, two-tailed sign test, $p<0.001$) exceeded pollinator residency times (figure 2*b*). Pollinarium reconfiguration times varied from 22 to 8100 s (mean = 952 s, median = 88 s). On average, reconfiguration times were 1.58 times longer than pollinator residency times.

4. DISCUSSION

The findings of this study provide strong empirical support for the idea that promotion of cross-pollination underlies the evolution of pollinarium reconfiguration. As predicted by Darwin's hypothesis, pollinarium reconfiguration times are both positively related to, and invariably exceed, pollinator visit times (figure 2*a*,*b*).

Self-pollination could also be prevented effectively by very long pollinarium reconfiguration times; however, reconfiguration times that greatly exceed pollinator residency times could be detrimental. In particular, cross-pollination opportunities could diminish if pollinia are lost quickly from pollinators and/or if pollinators leave a patch of conspecific plants before reconfiguration is complete. Thus mating

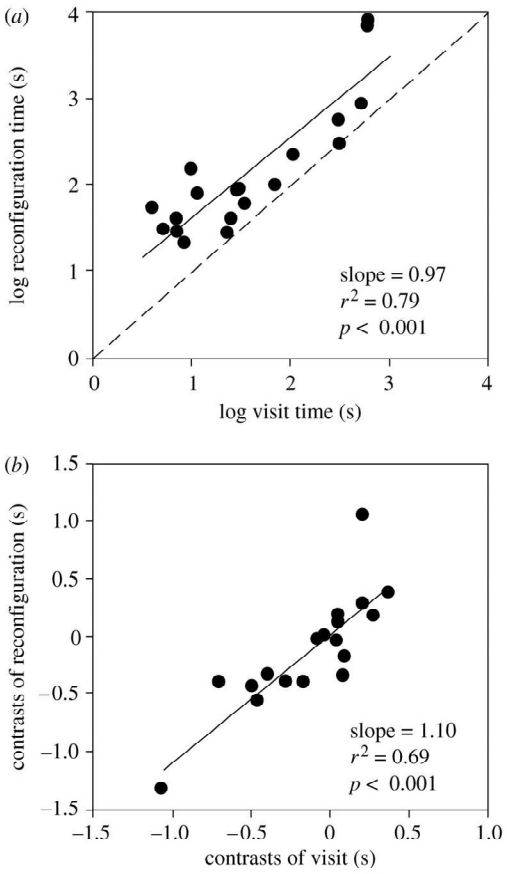

Figure 2. (*a*) There is a positive relationship between pollinarium reconfiguration and pollinator visit times. The data points are above the dashed line of unity, indicating that pollinarium reconfiguration tends to take place after the end of a pollinator visit. (*b*) The positive relationship between phylogentically independent contrasts of pollinarium reconfiguration and pollinator visit times.

opportunities should be maximized if pollinaria reconfigure shortly after pollinators have departed from the source plant, so that pollinia are ready for insertion into stigmas of the next plant visited. The positive relationship between reconfiguration and pollinator visit times (figure 2*a*,*b*) is consistent with this theoretical prediction.

The evolutionary lability in the timing of pollinarium reconfiguration is evidenced from the variation observed in this trait among two subspecies of *Eulophia parviflora*. One subspecies pollinated by slow-moving beetles has pollinaria that take an average of 100 seconds to reconfigure (figure 1), while the other subspecies pollinated by rapidly moving bees has pollinaria that reconfigure in just 28 s ($t_{111} = 17.53$, $p<0.0001$). The mechanism(s) behind the bending movements of both orchid and asclepiad pollinaria remain largely undocumented, but preliminary work by the authors supports the suggestion by Darwin (1862) that reconfiguration in some orchids involves differential drying of layers of tissue of the accessory structures attaching the pollinia to the pollinators. Darwin noted that tissue of the viscidium and base of the stipe may be important in

causing reconfiguration. In other orchids, tissue in the middle of the stipe appears to be responsible for reconfiguration. In some asclepiads, differential drying of tissue at the base of the translator arms may change the orientation of the pollinia (C. I. Peter, unpublished data).

Mechanisms that reduce the likelihood of self-pollination appear to be particularly prevalent in plant families in which pollen is aggregated as pollinia (cf. Johnson & Nilsson 1999; Harder & Johnson in press). Reconfiguration mechanisms have evolved at least four times (pollinarium bending in orchids and asclepiads, anther cap retention and pollinium shrinking in orchids) and perhaps many more times in these families. In this study, for example, the mechanism of bending in *Eulophia* and *Acrolophia* does not appear to be homologous with the mechanism found in the orchidoid species examined. While pollinarium reconfiguration is a mechanism confined to orchids and asclepiads, the remarkable evolutionary fine-tuning of this trait in response to pollinator visit times, as demonstrated in this study, conveys a broader message about the central role that pollinator behaviour plays in the evolution of plant traits that promote cross-pollination.

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[chapter 5]

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Reproductive biology of *Acrolophia cochlearis* (Orchidaceae): estimating rates of cross-pollination in epidendroid orchids

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• *Background and Aims* Pollen fates strongly influence mating success in plants but are difficult to quantify. By promoting foraging constancy in pollinators, floral rewards such as nectar may enhance the overall efficiency of pollen transfer. However, this can also lead to high levels of geitonogamy. Pollen fates were studied in *Acrolophia cochlearis*, a member of a terrestrial epidendroid orchid genus that includes both rewarding and deceptive species.
• *Methods* Pollinator observations were conducted. Pollen transfer efficiency (PTE), the proportion of removed pollinia deposited on stigmas, was measured in a large population at regular intervals throughout the 5-month flowering season. The level of cross-pollination in two populations was estimated from the percentage of seeds with embryos in naturally pollinated fruits.
• *Key Results Acrolophia cochlearis* (and a congener *A. micrantha*) produce minute but concentrated nectar rewards. Observations showed that *A. cochlearis* is pollinated exclusively by a solitary bee species, *Colletes claripes*. Although both sexes visited flowers, only males carried pollinaria. Overall levels of pollination and PTE of the rewarding *A. cochlearis* were much higher than in a deceptive congener, *A. capensis*. Seeds resulting from self-fertilization had a significantly lower probability of containing viable embryos than did those from cross-fertilization. This dichotomy in fruit quality was used to estimate that cross-pollination occurred in approx. 66 % of *A. cochlearis* flowers in a large dense population and approx. 10 % in a small sparse population. Traits of *A. cochlearis* that limit geitonogamy include pollinarium reconfiguration that exceeds the visit time of pollinators and rapid flower senescence following visitation.
• *Conclusions* Presence of a nectar reward in *Acrolophia cochlearis* results in high levels of PTE. It is estimated that approx. 33–90 % of fruits in natural populations arise from self-pollination in this species.

Key words: Reward, deception, pollen transfer efficiency, pollen tracking, geitonogamy, *Acrolophia cochlearis*, epidendroid orchid, Cape floral region.

INTRODUCTION

Nectar rewards encourage foraging constancy by pollinators (Goulson, 1999) and, as a result, should be expected to increase pollen transfer efficiency (PTE). PTE can be considered a population-level measure of the proportion of pollen removed from anthers that is subsequently deposited on conspecific stigmas (Johnson *et al.*, 2005; Harder and Johnson, 2008). In general, adaptations that increase PTE should enhance male fitness of individuals.

Nectar rewards can also increase the number of flowers that are visited by individual pollinators on a plant (cf. Johnson *et al.*, 2004). This can result in high levels of geitonogamy (Charlesworth and Charlesworth, 1987*b*; Johnson *et al.*, 2004; Jersáková and Johnson, 2006), which in turn may be detrimental to fitness because of the production of inferior, inbred offspring (Darwin, 1878; Charlesworth and Charlesworth, 1987*a*) and squandering of pollen grains and ovules that could otherwise be used for cross-pollination, processes known as pollen and ovule discounting, respectively (Barrett, 2002).

Patterns of pollen dispersal and geitonogamy are notoriously difficult to quantify. For plants that do not have aggregated pollen, tracking pollen movement is limited to the use of fluorescent powders that serve as pollen analogues (Snow *et al.*, 1996) as well as molecular markers that estimate parentage of offspring (e.g. Ritland, 1986; Eckert and Barrett, 1994; Galloway *et al.*, 2003; Herlihy and Eckert, 2004; Kruszewski and Galloway, 2006; Kropf and Renner, 2008). In orchids and asclepiads, packaging of pollen into pollinia allows for more direct tracking of pollen movement by labelling with unique microtags (Nilsson *et al.*, 1992), histochemical stains (Folsom, 1987; Peakall, 1989; Nilsson *et al.*, 1992; Salguero-Faría and Ackerman, 1999; Johnson *et al.*, 2005), coloured powders (Kropf and Renner, 2008) or radioisotopes (Pleasants, 1991).

This study focuses on the reproductive biology and patterns of pollen dispersal in *Acrolophia*, a poorly known and enigmatic genus of seven terrestrial species representing the only 'epidendroid' clade centred in the Cape (Linder and Kurzweil, 1999). *Acrolophia* is generally thought to be closely aligned to *Eulophia* (Linder and Kurzweil, 1999; although see Dressler 1993), a large African genus of deceptive terrestrial orchids. To date, nothing has been documented regarding the reproductive biology in the genus, although Russell (2005) observed high rates of seed set in

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A. cochlearis and consequently suggested that this species might be autogamous.

During the course of this study, it became apparent that *A. cochlearis* and *A. micrantha* produce nectar. This is an unusual condition among terrestrial epidendroid orchids in South Africa –nectar production has not been found in any of the many species of *Eulophia* examined (Peter and Johnson, 2006*a*, 2008; Peter, 2008). This provided a unique opportunity to study implications of nectar production for pollinia fates.

Although this study focuses on the pollination biology of *Acrolophia cochlearis*, it also considers aspects of the reproductive biology of *A. micrantha* and *A. capensis*. The six objectives were: (1) to determine the identity of pollinators and their interactions with flowers; (2) to determine the volume and concentration of nectar rewards; and (3) to determine the natural visitation rates to flowers and pollen transfer efficiencies of these rewarding and deceptive species. Given the production of nectar in *A. cochlearis* and numerous flowers on a plant, which both increase potential for geitonogamy, characteristics such as pollinarium reconfiguration that might function to reduce levels of geitonogamy were investigated (4). Finally, levels of inbreeding depression were quantified in a breeding system experiment (5) and the resulting difference in seed quality used to estimate levels of self- and cross-pollination in naturally pollinated flowers (6).

MATERIALS AND METHODS

Study sites

This study was undertaken mainly in a large population (approx. 1500 individuals) of *Acrolophia cochlearis* (Lindl.) growing along the edges of Mountain Drive, a dirt road along the crest of the Rietberg, south of Grahamstown, Eastern Cape, South Africa. A small sparse population of *A. cochlearis* growing on a sandy road cutting near the town of Kenton-on-Sea, 35 km south, south-east of Grahamstown was also examined. Additional observations were made on the ridge about 1·5 km to the south of Mountain Drive where the congeneric *A. capensis* is common and at the Coega rescue nursery near Port Elizabeth where plants of *A. cochlearis* and *A. micrantha* had been transplanted a short distance from a site being developed.

The study species

This study focuses on *A. cochlearis*, a relatively common orchid occurring from the Cape Peninsula in the west to the vicinity of East London in the east, with further sparse occurrences up the east coast of South Africa to about Richards Bay. This species produces extensive, branching inflorescences with numerous small non-resupinate flowers (Fig. 1A, B). Flowers are mostly drab and inconspicuous, with a white to pale cream labellum and a short sac-like spur. When numerous flowers are enclosed in a bottle to concentrate their scent, they have an obvious unpleasant sweet scent, although it is difficult to make out the scent of individual flowers or a few flowers on an inflorescence. For all measurements described below, only a single flower was sampled per plant.

Acrolophia micrantha is similar in many respects to *A. cochlearis*, although the flowers are resupinate and the labellum is larger and less 'clam-shell-like' (Fig. 2A). This species also has a short, broad sac-like spur.

In contrast, branched inflorescences of *A. capensis* (Fig. 2B) are more compact, and most of flowers open within a short period, remaining open for about 2 weeks. Sepals and petals are less spreading than in the case of the previous two species and form, with the base of the labellum, a tube surrounding the column. The central lobe of the labellum is large and showy, being bright white. Flowers of plants from around Grahamstown have a sweet honey-like scent.

Pollinators

Observations of pollinators were conducted at all three study sites on all three study species. However, only visits to *A. cochlearis* were observed at various sub-populations along Mountain Drive. Unlike in the case of many deceptive orchids where pollinators bearing pollinaria are typically captured on rewarding plants in the surrounding habitat, pollinators were only observed visiting *A. cochlearis* flower or patrolling clumps of these plants. A total of approx. 38 h was spent observing pollinators on *A. cochlearis* in 2003 and 2006/2007.

Insects were collected, killed using an ethyl acetate killing jar and mounted for identification. These are housed in the collection of the first author with vouchers housed in the entomology collection in the Albany Museum, Grahamstown (AMGS) and in the collection of Dr Michael Kuhlmann, Institute of Landscape-Ecology, University of Münster, Münster, Germany. Vouchers of plant species are housed in the Schonland Herbarium, Grahamstown (GRA).

Attempts were made to observe pollinators either visiting flowers or patrolling in the vicinity of *A. micrantha* and *A. capensis* plants, but these attempts were unsuccessful. Approximately 10 h were spent observing each of the other two species.

Nectar rewards

Nectar rewards for the three species were investigated. An examination of flowers from at least 15 *A. capensis* plants failed to detect nectar. The other two species contained minute quantities of nectar in the sac-like spurs. Flowers were therefore carefully broken open to reveal the nectar droplet. This was sucked up into a 4-μL micropipette. The length of the nectar column inside the pipette was measured using electronic calipers, and its volume then determined from this length relative to the length of the calibrated portion of the micropipette, which corresponds to a known volume. Because of the minute quantities of nectar it was not possible to measure nectar concentrations directly. Each sample was therefore diluted with a specific volume of water (also measured accurately with callipers in the same micropipette as the sample). The diluted nectar solution was then placed on the stage of an Atago 50 % sucrose refractometer and the concentration recorded. Knowing the dilution factor, it was possible to calculate the original nectar concentration.

Nectar was collected onto filter paper following refractometer measurements. These samples were used to determine the ratio of fructose : glucose : sucrose using high performance

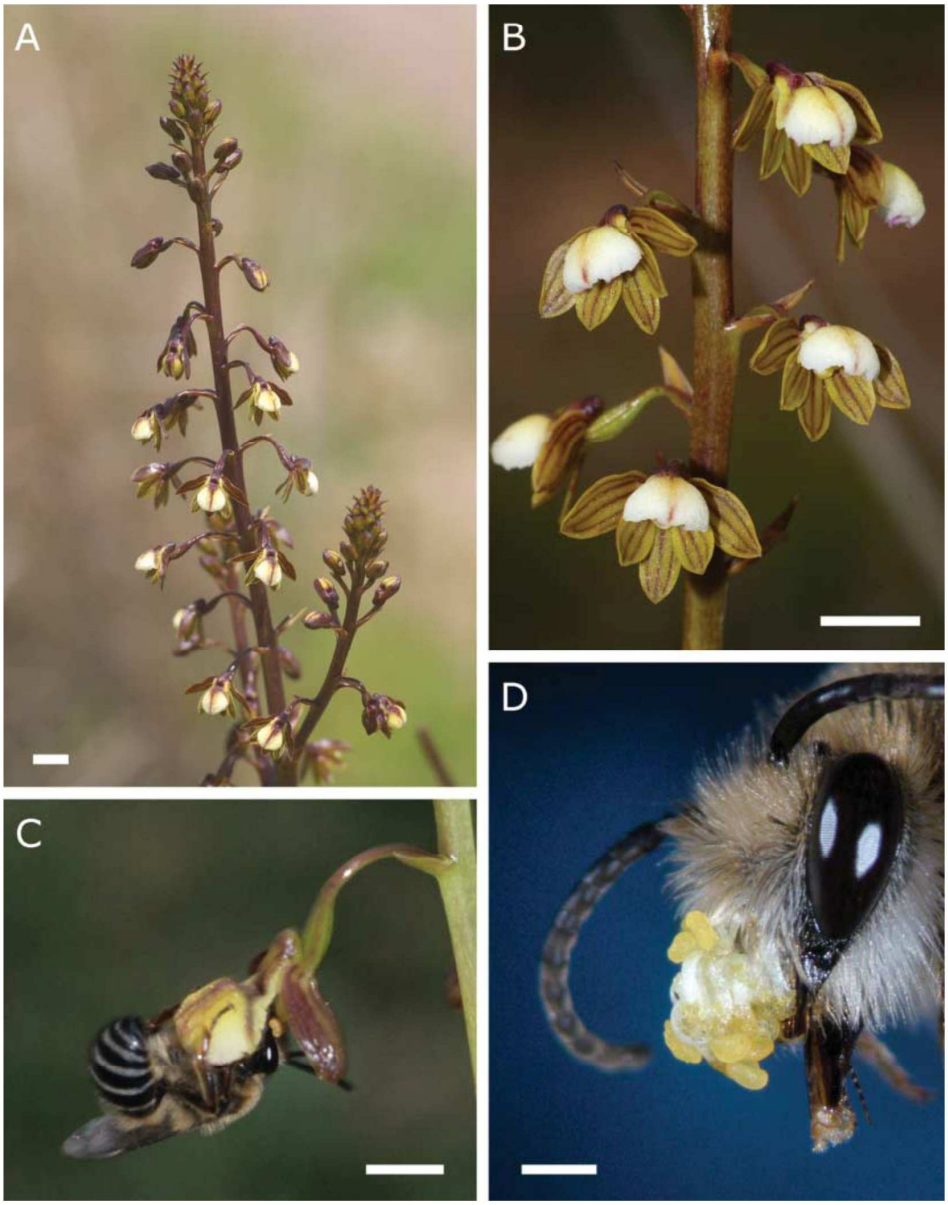

FIG. 1. *Acrolophia cochlearis* produces large branching inflorescences with numerous small flowers (A). The non-resupinate flowers are mostly drab with white to pale cream labellae (B). The pollinating male *Colletes claripes* bees assume an upside-down position when visiting flowers (C) and can amass substantial loads of pollinaria (D). Scale bars: (A–C) = 5 mm; (D) = 1 mm.

liquid chromatography as described by van Wyk *et al.* (1993). Because of minute volumes in each flower, samples from a number of individual flowers were pooled on the filter paper for later analysis.

Pollen transfer efficiency

Weekly surveys were conducted in a large population of *A. cochlearis* growing along the verge of a section of Mountain Drive. One flower was sampled randomly from each of approx. 160 plants per week. In addition, the numbers of plants not yet in flower and those that had completed flowering were counted.

Flowers collected were scored for pollinarium removal, pollinia deposition and failed visits (flowers with their anther cap disturbed but pollinaria not removed). From these data, as well as PTE, percentages were calculated of (*a*) plants in flower; (*b*) flowers with their pollinaria removed; (*c*) flowers pollinated;

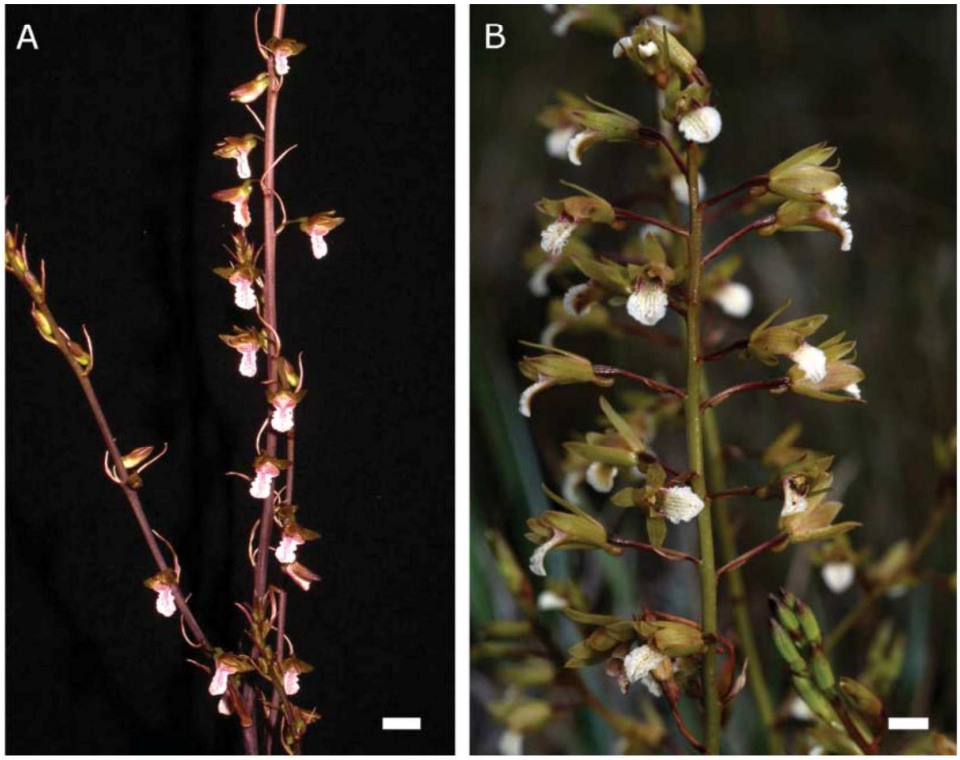

FIG. 2. Congeners of *Acrolophia cochlearis* examined in this study: (A) *A. micrantha* and (B) *A. capensis*. Scale bars = 5 mm.

(*d*) flowers showing any sign of legitimate visitation (removal, deposition or both); and (*e*) failed visits. PTE is the percentage of removed pollinia (pollinaria multiplied by two) that are deposited on stigmas (Johnson *et al.*, 2004). In addition, a survey of PTE and other measurements of pollination success, as listed above, were made on deceptive *A. capensis*.

Pollinarium reconfiguration and visit times

Due to the small size of pollinaria, bending rates of pollinaria in this species were measured under a dissecting microscope. Pollinaria were removed from flowers on the head of a pin and positioned above a protractor. Angles were then measured and plotted against the time after removal. Reconfiguration was assumed to be complete once the angle stopped changing. Visual inspections were used to determine duration of the pollinator visits to inflorescences.

Flower senescence

While examining natural visitation rates, it was noticed that flowers that had pollinaria removed started closing. To document this, pollinaria from flowers were removed and the span of the lateral sepals as well as width of the labellum measured before and 48 h after treatment. Control flowers on the same inflorescence were marked and measured at the same times. Percentage change data were arcsine-square root (angular) transformed to meet assumptions of normality required for ANOVA.

Breeding systems

Bagged flowers were cross-pollinated with pollinia from a plant >5 m away, self-pollinated, or left unmanipulated to test for auto-pollination. Hybrid crosses between *A. cochlearis* plants and a single individual of *A. micrantha* were also performed. Approximately 16 weeks after pollination we determined the proportion of flowers in the treatment groups that set fruit and percentage of seeds with viable embryos in each fruit. Percentage data were arcsine-square root transformed.

Estimating rates of geitonogamy in natural populations

We scored a sample of approx. 150 seeds in naturally produced fruit for the percentage of seeds with embryos. Using results of the breeding system as a reference, the proportion of seeds with embryos was used to determine whether a fruit resulted from self- or cross-pollination.

RESULTS

Pollinators

The observations indicate that *A. cochlearis* is pollinated exclusively by male *Colletes claripes* bees (Colletidae;

Fig. 1C). These bees accumulate large clumps of tiny pollinaria on their clypei (lower margin of the face), forming large yellow masses (Fig. 1D) clearly visible even on insects in flight. The average number of pollinaria per male bee was 14 with a range of 0–25 ($n = 8$). Besides the nine captured male bees, a further eight bees seen to be carrying pollinaria masses were not captured in the 2003 and 2006/2007 seasons. These were presumed to be male, given their colour.

Two female *C. claripes* bees were also observed visiting flowers, but neither carried pollinia. Females were relatively rarely encountered, whereas males apparently stay in the vicinity of a patch of plants, possibly for many days. However, as no insects were tagged, it is not possible to tell whether insects encountered at certain sites on consecutive days were the same individuals.

Both male and female bees when alighting on a flower assume an upside-down position and probe the short, sac-like spur for nectar (Fig. 1C).

A number of flies and occasional vespid wasps and honey bees were seen probing for nectar, but none of these insects removed pollinaria.

Nectar rewards

Acrolophia cochlearis has minute quantities of nectar with an average volume of only 0·037 μL (s.e. = 0·003; $n = 36$) per flower. The nectar is concentrated with an average sugar concentration of 90 % (s.e. = 0·467; $n = 32$). The average ratio of fructose : glucose : sucrose for a number of pooled samples from different plants in two separate analyses was 14 : 16 : 70. In *A. micrantha*, flowers contain 0·045 μL of nectar at a sugar concentration of 71 % ($n = 2$). The sugar composition of *A. micrantha* nectar was not analysed. None of the flowers from 15 individuals of *A. capensis* in the Rietberg population near Grahamstown produced nectar.

Pollen transfer efficiency

The flowering period in the *A. cochlearis* population extends over a 5-month period. It was possible to observe rates of flower visitation in the study population near Grahamstown at weekly intervals throughout the flowering period. By the fourth week of flowering, approx. 90 % of the plants were in flower, and flowering continued at this level for another 10 weeks before declining (Fig. 3A).

Overall visitation (flowers showing signs of pollen removal or deposition) increased steadily over the course of the flowering period (Fig. 3A), as did rates of pollinarium removal and pollinia deposition (Fig. 3B). PTE, on the other hand, fluctuated markedly throughout the flowering period (Fig. 3B). PTE in this species reaches 60 % in 2 weeks during the middle of the flowering period. High rates of PTE at the start of the flowering period may be a result of relatively small sample sizes early in the season and few flowering plants in the population. Pollination failure rates also fluctuated throughout the season but were highest in the first few weeks (Fig. 3A).

In contrast to high PTE for *A. cochlearis* in both 2003 and the average of the 2006/2007 season (Table 1), PTE in deceptive *A. capensis* was only 5·6 % (Table 1).

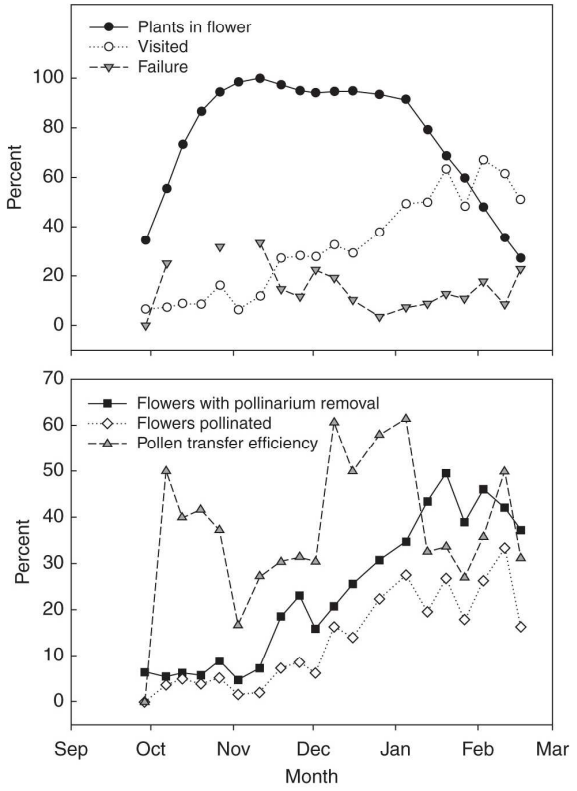

FIG. 3. Measures of *A. cochlearis* reproductive success tracked throughout the 5-month flowering period of this species.

Pollinarium reconfiguration

Freshly removed pollinaria remain erect, perpendicular to the surface of the clypeus and in a position where the pollinia are unlikely to contact the relatively large and exposed stigma. The pollinarium bends forward until it is apressed against the clypeus, a position where it is possible for the pollinia to be deposited on the stigma of a subsequent flower. This bending is comparable to the 'depression' of pollinaria Darwin described in a number of European orchids (Darwin, 1867) and takes 88 s on average (s.e. = 5·25; $n = 15$). The average visit time to inflorescences by male *Colletes* bees was 28 s ($n = 3$ visits by three bees to three inflorescences).

Pollinaria of *A. micrantha* undergo a similar reconfiguration, although reconfiguration time was not recorded in this species. Pollinaria of *A. capensis* on the other hand show no sign of reconfiguration, although anther cap retention similar to that described in *Eulophia foliosa* (Peter and Johnson, 2006*a*) has been suggested for this species in the west of its range (Russell, 2005).

Flower senescence

After removal of pollinia, flowers of *A. cochlearis* undergo a rapid change. Petals and sepals close around the column, and the labellum folds around its long axis and becomes stiff

TABLE 1. *Comparison of pollination success and pollen transfer efficiency in* A. capensis *and* A. cochlearis

	Date	No. of flowers	% flowers with pollinaria removed	% flowers pollinated	% Visited	% Failure	PTE (%)
A. capensis	1 December 2003	144	12·5	0·7	14·6	14·3	5·6
A. cochlearis	December 2003	184	70·7	47·3	82·6	3·3	38·5
A. cochlearis	2006/2007 season average	118	24·5	13·9	33·3	16·3	39·2

with less articulation at the mentum (Table 2). The labellum also rapidly changes from white or pale cream to a darker yellow-cream and ultimately to the brown of sepals and petals. Because of this rapid change, in many plants there are only one or two fresh flowers open per inflorescence branch at any one time.

Control flowers typically remained unchanged for longer than 48 h, but because random pairs of control and experimental flowers were chosen, some of them had been on the inflorescences longer than others and so after about 60 h some control flowers were beginning to close as a result of age.

Breeding systems

There was no difference in rates of fruit set between cross- and self-pollinated flowers (Table 3). However, the weight of capsules from self-pollination was significantly lower than that from cross-pollination and hybrid cross-pollination, as was percentage of fertile seeds in a fruit (Table 3). Bagged, unmanipulated flowers failed to set fruit, ruling out auto-pollination in this species.

Estimating rates of geitonogamy in natural populations

The different signals of fertile seeds produced by experimentally self- and cross-pollinated flowers (Table 3 and Fig. 4) were used to estimate the frequency of cross- and self-pollination in naturally pollinated flowers. Based on this experiment, fruits with $\leq$60 % of their seeds containing embryos were considered to result from self-pollination, and those with $\geq$61 % of their seeds containing embryos were considered to result from cross-pollination (Fig. 4). This analysis indicated that in a large and dense population, a high proportion (66 %) of naturally produced fruit is likely to be a result of cross-pollination (Fig. 4). In contrast, 94 % of naturally produced fruit in a small sparse population is likely to be the product of self-pollination (Fig. 4).

TABLE 2. *Percentage change in dimensions of flowers over 48 h following pollinarium removal or deposition*

Trait	Reduction in dimension (%)		
	Control (n = 12)	Pollinarium removed (n = 16)	Pollinia deposited (n = 6)
Span of lateral sepals	7·6[a]	33·9[b]	47·0[b]
Depth of labellum	4·4	7·1	5·5
Width of labellum	4·6[a]	26·8[b]	35·6[b]

Percentage change data were arcsine-square root (angular) transformed and compared using ANOVA. Homogenous groups were determined using a *post hoc* Tukey test and are indicated by the letters after the values.

DISCUSSION

Acrolophia contains both rewarding and deceptive species, in contrast to *Eulophia*, a large putatively related genus that appears to contain only deceptive species (Peter, 2008). Evolutionary transitions between reward and deception may not be uncommon in orchids, as there have been reports of both rewarding and deceptive species in a number of orchid genera (van der Cingel, 1995, 2001; Johnson *et al.*, 1998). Uncovering the evolutionary basis for these transitions is therefore a major challenge for plant reproductive biology.

Nectar is expected to increase not only overall visitation rates, with pollinators visiting more flowers in a patch compared to deceptive species, but also overall PTE, as pollinators should show greater foraging constancy. Indeed, overall pollination success and PTE in the rewarding species *A. cochlearis* is much higher than in the deceptive *A. capensis* (Table 1 and Fig. 3B) and in the exclusively deceptive species of *Eulophia* examined to date. Although PTE in the *A. cochlearis* population exceeded 60 % in some weeks, the average for 13 *Eulophia* species surveyed ranged from 11 % to 28 % (Peter, 2008).

A more comprehensive comparison of PTE between the rewarding and deceptive species of *Acrolophia* will be enlightening. Utilizing the survey of rewarding and deceptive species listed by van der Cingel (1995, 2001), it is possible to identify at least 14 genera containing both rewarding and deceptive species. Direct comparisons of PTE among rewarding and deceptive species, however, could be confounded by other factors, such as pollen vector. For this reason it will probably be most profitable to either search for rewarding and deceptive sister taxa with common pollinators or to attempt to experimentally add nectar to whole populations of deceptive taxa.

The evidence presented in this study indicates that *A. cochlearis* has a highly specialized pollination system involving a single bee species in the genus *Colletes*. These short-tongued bees feed on the concentrated nectar with a sucrose to hexose ratio of 2·33. Baker and Baker (1990) surveyed over 700 species and found that short-tongued bees prefer hexose-rich nectars. However, Schmidt-Lebuhn *et al.* (2007) found that nectars of bee-pollinated species in Acanthaceae are concentrated and dominated by sucrose. Similarly, Petanidou (2005) found that nectars of bee-pollinated Mediterranean species across a number of families are

TABLE 3. *Results of controlled pollination experiments to determine the breeding system of* A. cochlearis

	Cross-pollinated ($n = 11$)	Self-pollinated ($n = 7$)	*A. cochlearis** × *A. micrantha* ($n = 6$)	Bagged and unmanipulated ($n = 22$)
Fruit set (%)	100	100	100	0
Mean capsule and seed weight (g)	$0{\cdot}05 \pm 0{\cdot}01^{a}$	$0{\cdot}02 \pm 0{\cdot}01^{b}$	$0{\cdot}06 \pm 0{\cdot}01^{a}$	–
Percentage of seeds with embryos†	$90{\cdot}4 \pm 3{\cdot}1^{a}$	$26{\cdot}2 \pm 9{\cdot}3^{b}$	$89{\cdot}9 \pm 2{\cdot}0^{a}$	–

Values are means (± s.e.).
Homogenous groups were determined using a *post hoc* Tukeys test and are indicated by the letters after the values.
*Female parent.
†Percentage data were arcsine-square root transformed and compared using ANOVA.

dominated by sucrose. These contrasting results suggest that sugar composition is not a particularly critical aspect for bee pollination.

To date, little attention has been given to the biology of *Acrolophia* besides curiosity over the unusual 'black' flowers of *A. ustulata* (Kurze and Kurze Hilde, 1990). Russell (2005) observed high rates of fruit set in *A. cochlearis* in the Western Cape and suggested that rain-assisted autogamy might be at play in this species as has been suggested for *Oeceoclades maculata* (González-Díaz and Ackerman, 1988). However, from the data presented here, high rates of capsule production seem to be a consequence of high rates of visitation by bees. Because *Acrolophia* species do not have brightly coloured flowers, it is likely that future investigations will show that fragrance plays a key role in attraction of bees.

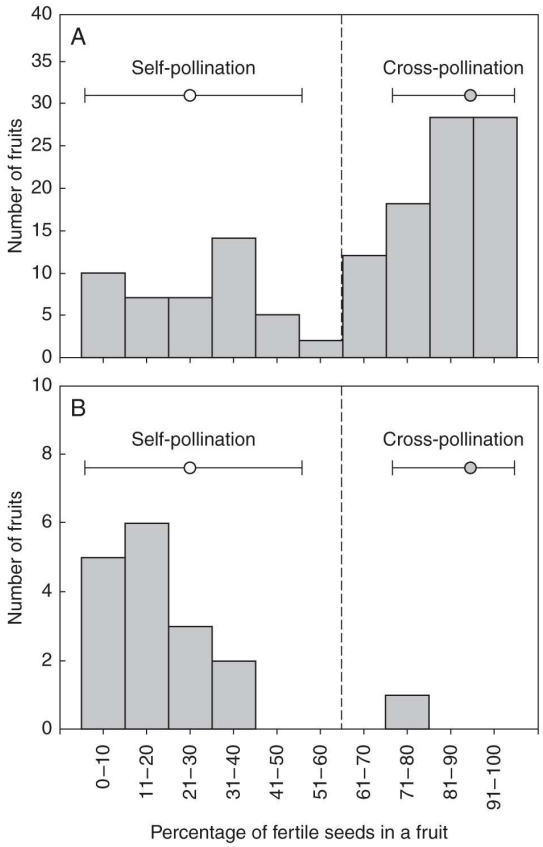

FIG. 4. Survey of seed quality in naturally produced fruit from a large, dense population (A, $n_{fruit} = 131$, $n_{plants} = 41$) and a small, sparse population (B, $n_{fruit} = 17$, $n_{plants} = 4$). Circular symbols and horizontal bars indicate mean and range, respectively, for experimental fruits produced by self- and cross-fertilization (see Table 3). The dashed line separates fruits estimated to be due to self-fertilization from those estimated to be due to cross-fertilization.

The combination of nectar rewards with large numbers of flowers on plants of *A. cochlearis* (and *A. micrantha*) must greatly increase the risk of geitonogamy. *Acrolophia cochlearis* and *A. micrantha* both have a pollinarium reconfiguration mechanism and, at least in *A. cochlearis*, there is evidence that this reconfiguration can partly protect the species from geitonogamy as reconfiguration takes longer than the average visits observed (see also Peter and Johnson, 2006*b*).

The amassing of pollinia that was observed on many of the captured bees (Fig. 1D) could have important implications for the efficiency of the pollinarium reconfiguration system, as well as the likelihood of pollination itself. On the other hand, should a pollinator bearing a large number of pollinaria (e.g. up to 40 pollinia) visit a plant, the odds of geitonogamy would be reduced simply because freshly removed self-pollinia are in a minority among the many pollinaria carried. The relatively open architecture of *A. cochlearis* flowers allows the short-tongued bees to probe flowers in variable positions such that most of the pollinia on the surface of the half sphere of the pollen mass have an equal chance of being deposited on the stigma of the flower.

Finally, the rapid senescence of visited flowers (Table 2) implies that, in many instances at the height of flowering, there are relatively few flowers open at any one time and often only one fresh flower open per inflorescence branch. This phenomenon reduces the potential for geitonogamy (Harder and Johnson, 2005), but only when visitation rates are high. Earlier in the season, when visitation rates are lower (Fig. 3) there are more virgin flowers open on an inflorescence at one time (e.g. Fig. 1A, B), and geitonogamy might then be more prevalent. The finding that flowers rapidly senesce following removal of pollinaria differs from previous studies of orchids that showed that deposition of pollinia, but not pollinarium removal, has a strong effect on senescence

(Proctor and Harder, 1995; Luyt and Johnson, 2001). Senescence of flowers following removal of a pollinarium could result in loss of female function and suggests strong selective pressure against having a large number of flowers open simultaneously in order to minimize pollen discounting.

Direct assessment of rates of self-pollination in *A. cochlearis* would be desirable. Ideally this would take the form of direct pollinia tracking using histochemical or powder stains (Peakall, 1989; Kropf and Renner, 2008) or microtags (Nilsson *et al.*, 1992), but given the small size of pollinia and presence of an anther cap that completely encloses the pollinia neither of these two established methods is feasible in this species. An alternative approach to estimating rates of selfing and outcrossing entails germinating seeds and using allozyme markers to estimate the degree of inbreeding (Eckert and Barrett, 1994; Ortiz-Barney and Ackerman, 1999). However, this technique is slow, costly and unreliable in the case of orchids that require sterile germination of seeds on nutrient media (Thompson *et al.*, 2001).

Given the difficulties in making direct measurements of self-pollination in *A. cochlearis*, an alternative method based on fruit quality was used. The different ratios of fertile to infertile seeds produced by self- and cross-pollinated capsules (Table 3) allow the parentage of naturally produced fruit to be determined with a high degree of confidence. This method does not involve manipulation of flowers and is much easier than other techniques to implement. Differences between self- and cross-fertilized fruits in the proportion of seeds containing embryos have been shown in many studies of orchid species (Tremblay *et al.*, 2005; Jersáková *et al.*, 2006). The basis for this is not well understood, but it is believed to result from severe inbreeding depression at the embryo development stage.

Variation in genetic load among populations may limit the applicability of the method to the same populations in which experimental cross- and self-pollinations were performed. Thus, the inference that levels of self-pollination were much higher in a small population of *A. cochlearis* because of the distribution of fruit quality in that population (Fig 4) should be considered tentative because the effect of self-pollination on the percentage of seeds with embryos was not quantified in that population.

The technique only works when selfed and out-crossed fruits clearly differ in their proportion of seeds with embryos, a situation that is clearly not met in all orchids (cf. Tremblay *et al.*, 2005; Jersáková *et al.*, 2006). It is also obviously not suited to orchids with massulate pollinia where stigmas can receive a mixture of self- and cross-pollen. Interspecific pollinia movement may also obscure the signal, being confused with either self- or out-crossed pollination (cf. Table 3; Kallunki, 1981; Borba *et al.*, 2001), although this is less of a problem in species with highly specialized pollination systems. Despite these limitations, this technique should be applicable to many epidendroid orchids and, thus, to a large proportion of the family.

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Pollinator-driven divergence

[chapter 6]

A pollinator shift explains floral divergence in the *Eulophia parviflora* complex

ABSTRACT Floral diversification driven by shifts between pollinators has been one of the key explanations for the radiation of angiosperms. According to the Grant-Stebbins model of pollinator-driven speciation, these shifts result in morphologically distinct "ecotypes" which may eventually become recognizable as species. I identified two forms of the southern African orchid *Eulophia parviflora* (Lindl.) A.V. Hall that differ in distribution, floral morphology, scent chemistry and phenology. Each form is pollinated by a different insect species, and thus represents a distinct ecotype. The coastal form which has long spurs, floral scent dominated by terpenoid compounds and early flowering, is pollinated exclusively by the long-tongued bee *Amegilla fallax* (Anthophorinae; Apidae), while the inland form with short-spurs, floral scent dominated by aromatic compounds, and late flowering is pollinated exclusively by the beetle *Cyrtothyrea marginalis* (Cetoniinae; Scarabaeidae). Choice experiments in a y-maze olfactometer showed that beetles are preferentially attracted to the scent of the short-spurred form. A spur-shortening experiment showed that long spurs are required for effective pollination of the bee-pollinated form. Although it was hypothesized that divergence occurred across a geographical pollinator gradient, plants of the long-spurred coastal form were effectively pollinated when transplanted to an inland locality. Thus, the underlying geographical basis for the evolution of ecotypes in the *E. parviflora* complex remains uncertain, although early flowering in the long spurred form to exploit the emergence of naïve bees may restrict this form to coastal areas where there is no frost that would damage flower buds. Later flowering of the short-spurred form coincides closely with the emergence of the pollinating beetles following winter frosts.

INTRODUCTION

Adaptation to pollinators is generally considered to be the primary reason for floral diversification in plants (Soltis *et al.* 2005). In a conceptual model first developed by Grant and Grant (1965) and Stebbins (1970), pollinator-driven diversification begins with adaptation by plants to their most effective pollinators in a local region. Given a geographical mosaic of pollinator availability, this would result in divergence of "pollination ecotypes" within a species (Herrera *et al.* 2006). Ultimately, this process could result in speciation if allopatric forms become sufficiently morphologically distinct, or when forms become reproductively isolated enough to coexist without genetic dissolution through hybridization (Johnson 2006). The latter aspect of the model is especially appealing to adherents of the biological species concept because adaptive shifts between pollinators can have pleiotropic consequence for reproductive isolation (Fulton & Hodges 1999, Bradshaw & Schemske 2003). However, by selective modification of some of the most conspicuous traits of plants, pollinators are also responsible for many of the characters that are conventionally used to diagnose species (Grant 1949, Johnson 1996).

The Grant-Stebbins model, as it was termed by Johnson (2006), is well supported by microevolutionary studies of selection imposed by pollinators (Conner 2006, Morgan 2006), as well as macroevolutionary studies that show links between shifts in pollination system and cladogenesis (Givnish & Sytsma 1997, Johnson *et al.* 1998, Soltis *et al.* 2005). However, an intermediate stage, the evolution of local pollination ecotypes within species remains very poorly documented (Herrera *et al.* 2006, Johnson 2006).

The most basic evidence for pollination ecotypes consists of correlations between floral forms and particular pollinators. The most frequently documented of these "trait-environment" correlations involve ecotypes with differing flower tube length and pollinators of correspondingly variable tongue length (Robertson & Wyatt 1990, Johnson 1997, Johnson & Steiner 1997, Boyd 2004). There is also some evidence that intraspecific variation in floral scent chemistry can be associated with different pollinators (Pellmyr 1986, Johnson *et al.* 2005). Flowering phenology is another trait that has been investigated with respect to pollinator shifts among forms within a species (Herrera *et al.* 2002). It should also be noted

that several studies have not found clear evidence that floral forms correspond to a geographical mosaic of pollinators (Robertson & Wyatt 1990, Herrera *et al.* 2002, Herrera *et al.* 2006).

Very few of the abovementioned studies of pollination ecotypes include evidence that traits that characterize putative ecotypes arose through selection by pollinators. Such evidence can be derived from experiments where pollinators of one of the ecotypes are presented with an array of all the ecotypes to determine foraging preferences or pollination effectiveness. Arrays can be assembled under laboratory conditions and presented to captive insects (Galen 1989) or arranged in the field by means of transplant experiments (Robertson & Wyatt 1990) or by manipulating flowers within a population (Johnson & Steiner 1997).

While investigating the pollination biology of *Eulophia parviflora*, I encountered two forms that appeared to differ in floral morphology, floral fragrance, and flowering time. I hypothesised that these differences reflect adaptations to different pollinators, and thus constitute pollination ecotypes. To test this hypothesis, I tested the following predictions: 1) morphology, scent chemistry and flowering times are quantitatively different between the forms, 2) floral traits of each form are correlated with each other (i.e. constitute syndromes) and are geographically structured, 3) pollinators differ between the two forms, 4) pollinators discriminate between the two forms when offered a choice, 5) floral traits that differ among forms influence the effectiveness of pollinators.

MATERIAL AND METHODS

Study species

Eulophia is a large genus of terrestrial orchid found predominantly in Africa. These plants grow sympodally with subterranean tubers. Each year the tubers produce a new vegetative shoot as well as an inflorescence from the base of the vegetative shoot. All species of *Eulophia* that I have examined are deceptive and neither of the two forms described here reward their pollinators.

Eulophia parviflora, described by Hall (1965, p149; Fig. 1) as a "rather variable species," occurs in grasslands of the eastern parts of South Africa (Fig. 2). While investigating the

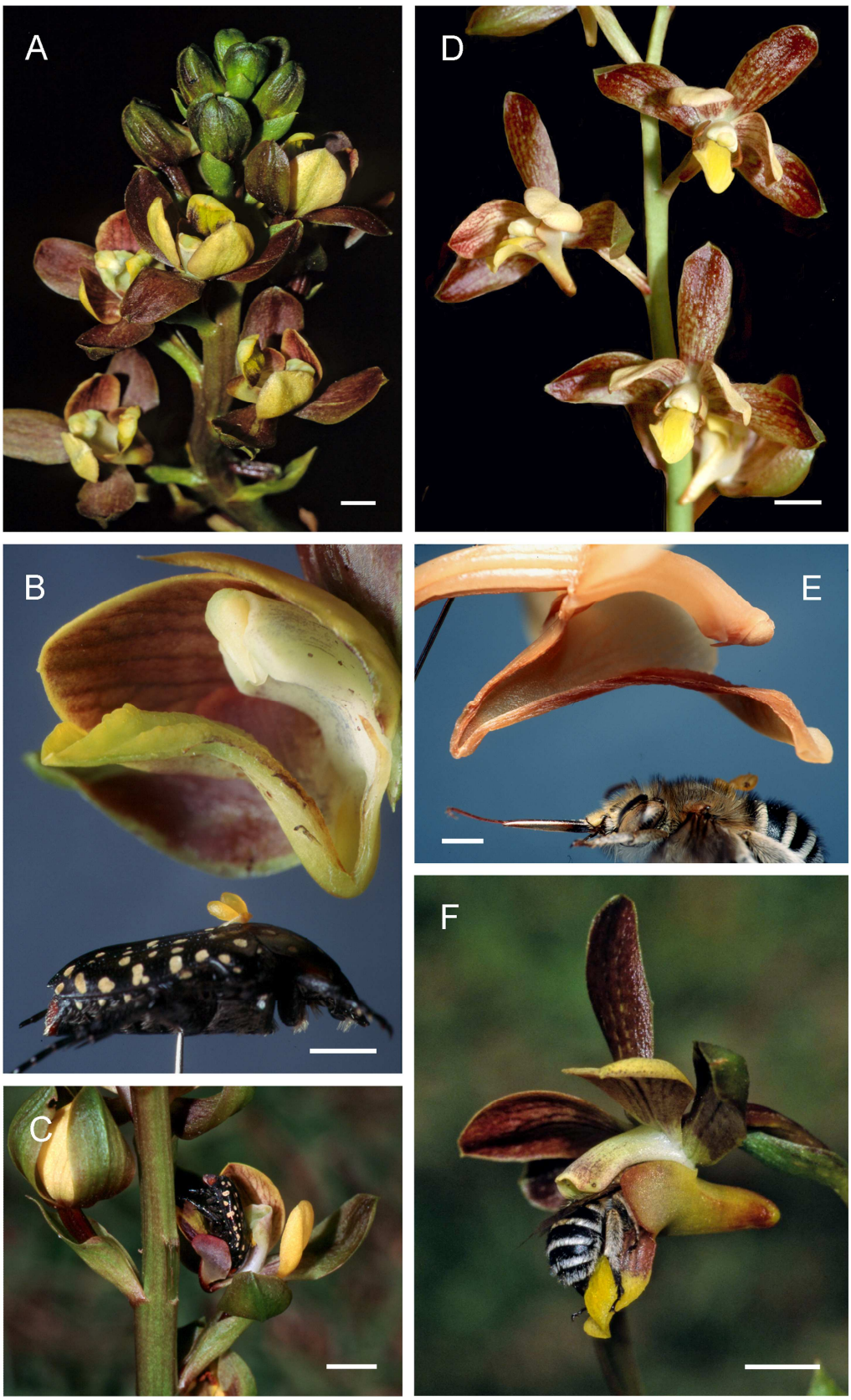

Figure 1A-F: Floral morphology and pollinators of *Eulophia parviflora*. A. short-spurred morph. B Dissected flower of the short-spurred form showing the short wide spur that accommodates the short blunt head of pollinating *Cyrtothyrea marginalis* beetles. C. *Cyrtothyrea marginalis* visiting a flower of the short-spurred form. D. Long-spurred form of *Eulophia parviflora*. E. Dissected flower of the long-spurred form showing the relatively long and slender spur that accommodates the long proboscide of *Amegilla fallax*. F, *Amegilla fallax* visiting a flower of the long-spurred form. Bars = 5 mm except B & E = 2 mm.

pollination biology of this species, I noticed that it is comprised of two distinct forms. One form has tall dense inflorescences with large numbers of non-resupinate flowers with short spurs (Fig. 1B). This form, "the short-spurred form" appears to flower later in the season, have relatively well developed vegetative shoot and a distinct sweet cherry scent. In contrast the early flowering "long-spurred form" has shorter inflorescences made up of many fewer resupinating flowers with long spurs (Fig. 1A) and an attractive "lily-of-the-valley" scent. At anthesis, the vegetative shoots have rarely emerged from the ground.

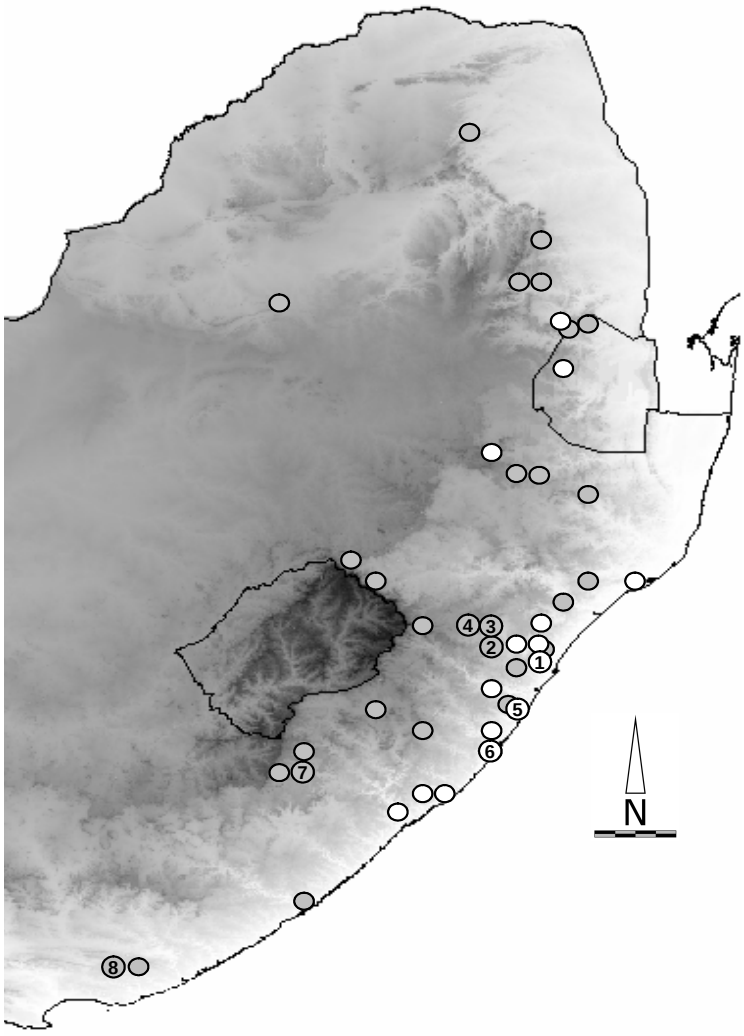

Figure 2: Distribution of the short- (●) and long-spurred (○) forms of *E. parviflora* in South Africa. Study sites in KwaZulu-Natal include 1) Krantzkloof Nature Reserve (a) and the two ends of Stockville valley (b and c) both in Kloof near Durban and separated by a distance of c. 9 km; 2) Victoria Country Club on the outskirts of Pietermaritzburg; 3) Umgeni Valley Nature Reserve near Howick; 4) road verge near Balgowan in the KwaZulu-Natal midlands. Additional observations were made at 5) Vernon Crookes nature reserve and 6) Umtamvuna nature reserve. In the Eastern Cape Province, sites include 7) the outskirts of the town of Maclear and 8) the Rietberg, near Grahamstown. Darker shades of grey represent increasing altitude. Bar = 100 km.

Study sites

Populations of both forms of *E. parviflora* were observed at various sites in KwaZulu-Natal and the Eastern Cape (Fig. 2) between September 2000 and October 2002. Additional observations of pollinators visiting the short spurred form were made at Krantzkloof in September 2004 and Grahamstown in 2006.

Phenetic analysis

Floral and vegetative characters

A large number of floral and vegetative characters were measured and are listed in Appendix A. The majority (58) of these measurements are quantitative characters, although three binary state characters are included (Table S1). I also include a number of ratios which describe the shape of a number of floral parts such as petals.

Colour characters

The colour of various flower parts (sepals, lateral petals and labellae) were measured with an Ocean Optics S2000 spectrophotometer. An Ocean Optics Mini–D2T (Tungsten-Deuterium-Halogen) light source was used to illuminate the sample. The reflection probe (UV/VIS 400 micron) was orientated at 45° to the surface of the floral part being measured.

Colours were summarised using the Endler segment classification method (Endler 1990). A colour model based on the spectral sensitivity of the pollinator's colour receptor would be a preferable method for summarising colour and such models are available for bees. However there is little information about the sensitivity of beetle receptors and there is evidence that some species respond to the red part of the colour spectrum (Briscoe & Chittka 2001). Bees have limited sensitivity to the red part of the spectrum and as a result, models such as that of Chittka (1992) are probably inappropriate methods for describing the colour of flowers pollinated by beetles.

The Endler (1990) segment classification takes the integral of light reflected from floral part and the light incident on the sample (the D65 norm-function in this case) for each of four equal segments between 300 and 700 nm. These values are divided by the integral for the entire spectrum of interest (300 – 700 nm) to separate colour from brightness and subtracted from one another to determine values for colour "opponents". The value for the medium-short wavelength segment is subtracted from the long wavelength to give a long-medium

(LM) opponent and the short wavelength segment is subtracted from medium long segment to determine the medium short (MS) opponent.

Analysis

Morphological measurements were recorded from living plants or material in 70% ethanol. Colour measurements were recorded from freshly harvested flowers and values for the LM and MS opponents for each flower part measured were included as characters. A total of 84 characters were measured for 46 and 47 specimens of the short- and long-spurred forms respectively (Table S1). Missing data accounted for 1.2% of the characters scored. Data were analysed using PCA in NTSYS-pc (Version 2.0; Rohlf 2000). Quantitative data were first log-transformed (Jolicoeur 1963, Humphries *et al.* 1981) and then a correlation matrix, calculated (SIMINT). The data were not standardised using the STAND module as this is accomplished by the correlation matrix (Somer 1986, James & McCulloch 1990). The first two principle components were then extracted from the correlation matrix (EIGEN) and plotted against each other. Eigenvalues are given in Table S2.

Distribution, flowering phenology and flight times

Distribution and flowering phenology data was collected from specimens from a variety of herbaria including NU, NH, PRE, BOL, GRA and K. In addition, my field observations were included, with each observation representing an opportunity where a herbarium specimen could have been collected. Distribution data that were accurate to at least one minute of latitude and longitude was used to extract start and end dates for frost periods from the climatic surfaces of (Schulze *et al.* 1997).

Flight times of the pollinating insects were determined from specimens in AMGS, TMSA and SANC as well as from Eardley's (1994) revision. Distribution data for the two pollinator species are given by Eardley (1994) and Holm & Marais (1992).

In all cases, dates were numbered consecutively with 01 July being the first, and 30 June the 365th day of the season to span the austral summer.

Insect pollinators

A total of 109 and 63 hours were spent in the field examining pollinators of the long- and short-spurred forms respectively. Observations at each site ranged between 1 hour at the

Maclear site and a total of about 67 hours at the two Stockville Valley sites. At all sites, besides Maclear, observations were made on two or more days. All insects found on the inflorescences and other possible pollinators (primarily Hymenoptera, Coleoptera and Diptera) visiting other plants in the vicinity where collected and inspected for pollinaria or viscidia. Where pollinators were observed visiting flowers, the duration of the visits to individual inflorescences were recorded. Voucher specimens are lodged in the Albany Museum and the personal collection of the author.

Pollinarium reconfiguration

Pollinaria in this, and most other species in *Eulophia*, undergo a reconfiguration following removal from the flower. In these two forms, this reconfiguration entails the stipe of the pollinarium bending through approximately 160°. Figure 1 of Peter & Johnson (2006b [Chapter 4]) illustrates this mechanism in the short-spurred form. Because this change is relatively quick and the end point is obvious in these two forms of *E. parviflora*, the pollinaria were removed with a finger nail and time taken in seconds for the pollinarium to complete its reconfiguration was recorded.

Scent analysis

Headspace scent samples of three inflorescences of each form were collected in the field and the sites and dates of collection are given in Supplementary Table S4. Inflorescences were enclosed in a glass bell jar and headspace air was drawn through a filter containing 3 mg of Porapak for approximately 6 hours (at approximately 2 l/hr). Scent compounds were eluted in a 5:1 hexane:acetone mixture and their relative abundances were determined using GC-MS according to the method of Kaiser & Tollsten (1995).

Freshly harvested flowers were immersed in neutral red dye to locate osmophores. Due to the fact that both forms of *E. parviflora* are deceptive and no nectar was found in any of the flowers, stained parts of the flower are likely to indicate the position of the osmophores.

Scent choice experiments

I constructed a "Y-shaped" olfactometer with small computer fans blowing ambient air through stainless steel scent chambers into each of two arms of the perspex olfactometer. Each of the arms was 150 mm long with a common base arm of 150 mm. The clear perpex tubing was 60 mm in diameter (Fig. S1). Pollinators were introduced to the olfactometer and

their choices recorded. Although this experiment was attempted using both bee and beetle pollinators, bees did not show motivation to move within the confines of the olfactometer. Insects for these experiments were collected from sites were the plants were not present to avoid prior conditioning by the deceptive flowers but within the general range of the plants. Insects were kept in a cool dark cage for 24 hours before the experiment and were not fed. Experiment were conducted mid morning to avoid the hottest part of the day when the activity of these insects is lower. Beetles were used no more than twice in an experiment.

Choice experiments were conducted in a greenhouse made with opaque fibreglass sheets in order to achieve diffuse lighting. The alignment of the olfactometer in relation to the sun was critical even within the greenhouse. The axis of the olfactometer was aligned directly at the sun and I made sure that 50% of beetles selected each of the two arms when no scent samples were included (Fig. 6A).

Once the olfactometer was correctly aligned, flow rates were balanced and beetles were not showing a preference for either arm of the olfactometer in the absence of odour cues, fresh flowers producing scent were introduced to one of the two arms (selected randomly), the flow rates were re-balanced, the beetles introduced and their choices recorded. I tested two combinations of scents. In the first experiment, I tested the scent of the short-spurred form of the orchid against a blank control with no odour. In the second, I tested the scent of the short-spurred flowers against the scent of the long-spurred form.

Visitation rates and pollen transfer efficiency

All flowers on a number of inflorescences from the populations sampled were scored for pollinaria removal and pollinia deposition. I also determined what number of flowers showed any sign of visitation as well as flowers that had their anther caps disturbed which represents a failed visit. Finally I calculated the pollination transfer efficiency. This is the percentage of removed pollinia (removed pollinaria multiplied by two) that are deposited on the stigmas (Johnson *et al.* 2004).

Translocation experiment

A translocation experiment was conducted to determine if orchids would be pollinated effectively if moved outside of their natural distribution range. A large population of the long-spurred form flowered in a firebreak shortly before this area was due to be burnt.

I therefore harvested these inflorescences without disturbing the below ground tuber-stock and used these for a translocation experiment. Inflorescences were inspected and flowers showing signs of visitation were removed. The inflorescences were then positioned at a natural height in the grass canopy with their cut ends immersed in water in glass pill-vials. Inflorescences were assigned randomly to two sites, one within the natural distribution of the long-spurred form, the second about 60 kilometres outside the natural distribution of the long-spurred form, but in a population of the short-spurred form. Inflorescences were left for seven days before the flowers were inspected for signs of pollen removal and deposition.

Given the widespread distribution of the short-spurred form (Fig. 2), it was not feasible to do a similar translocation experiment with this form. I did however record the visitation rates to plants of the short-spurred form at each of the two locations to which the long-spurred form was translocated.

Spur-shortening experiment

To investigate the role of floral morphology in pollinator effectiveness, I shortened the spurs of the long-spurred form at the Stockville Valley site by approximately 50%. Because of the fleshy nature of the spurs of these flowers, finger pressure was used to press the spur flat and then a small plastic clamps were used to hold the spur closed. Pairs of plants growing in proximity to one another were assigned either to the shortening treatment or left unmanipulated to serve as a control. Flowers on both treatment and control plants were first examined for any signs of visitation and visited flowers removed before the treatment performed. Flowers were left for five days and then inspected for signs of pollen removal or deposition. The proportion of flowers with pollinia removed was compared using a paired t-test after the data were arcsine square root transformed.

Breeding system

Inflorescences were bagged to exclude pollinators and then flowers were self-pollinated, cross-pollinated or left unmanipulated to test for auto-pollination. Breeding systems were conducted at the Victory Country Club and Stockville Valley sites for the short and long-spurred forms respectively. In addition, reciprocal crosses were made between flowers of the two forms at each of the two sites.

RESULTS

Phenetic analysis

PCA analysis of the various characters measured, confirm the presence of two distinct forms (Fig. 3). This analysis identifies one individual that might be intermediate between the two forms. The majority of these characters show significant differences between the two forms (Supplementary Table S2).

Spur length is one of the most obvious characters that separate the two forms (Fig. 1B & 1E). Spur length shows a clear bimodal distribution with no overlap between the two forms (Fig. 4) identified *a priori* based on the remaining suite of characters (Supplementary Table S2).

Colour

The colours of the two forms are nearly identical and overlap extensively (Supplementary Fig. S2). The adaxial surface of the lateral petals is the most variable colour ranging from light creamy-yellow to dark brick-red with many combinations of mottling of these two colours explaining the variation in measured points (Supplementary Fig. S2A). This variation is not specific to either form.

Distribution, flowering phenology and flight times

The long-spurred form is found primarily at lower altitudes along the coast of KwaZulu-Natal, while the short-spurred form is found at higher altitudes through the Eastern Cape, KwaZulu-Natal and Mpumalanga Provinces (Fig. 2). There is limited overlap of the two forms in the vicinity of Durban where the short-spurred form is found at lower altitude (Fig. 2, site 1). In the north-east part of the range, the distributions of the two forms also overlap with the long-spurred form found at higher altitude in the mountains of Mpumalanga and Swaziland.

The long-spurred form flowers significantly earlier than the short spurred-form in the central part of the distribution ($t_{84} = 10.0$, $p < 0.0001$; Fig. 5). There is some overlap in the flowering phenology of the two forms, particularly when the long-spurred form flowers later in the season (probably in response to late fires).

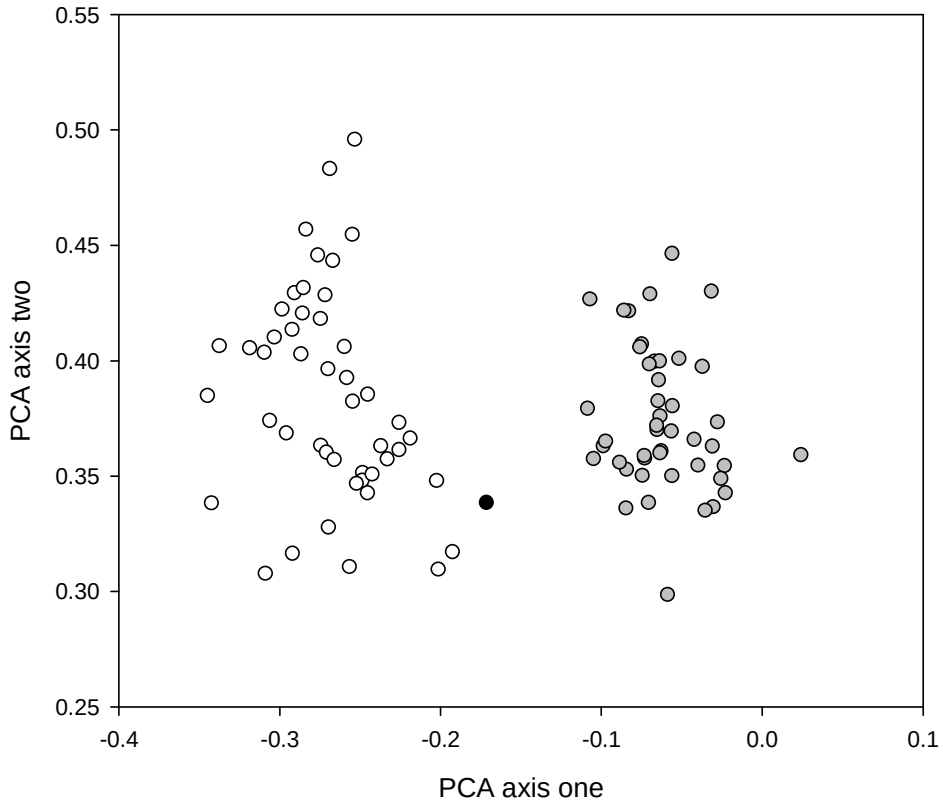

Figure 3: Phenetic analysis of the morphological characters of the long (○, n = 47) and short (●, n = 46) forms of *E. parviflora*. A possible hybrid or intermediate is also indicated (●).

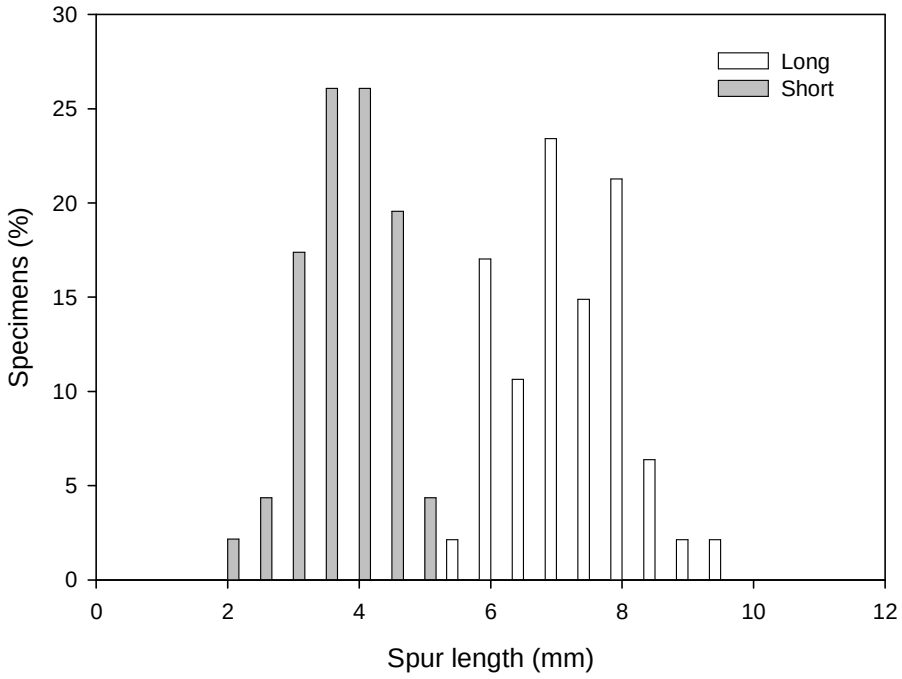

Figure 4: The bimodal distribution of spur length in *Eulophia parviflora* corresponding to the long- (n = 47) and short-spurred (n = 46) forms identified *a priori* using other morphological characters.

In both forms, flowering of the orchids corresponds with the emergence of their respective pollinators (described below), although the flight period of the pollinators extends for a much longer period than flowering of the orchids (Fig. 5 A & D). While there are a few unusual flowering records, flowering of both forms typically commences thirty days after the last day of frost in their respective distributions (Fig. 5 B & C). Frost is rare within the range of the long-spurred form, and restricted to only a few days at the height of winter. Frost is more widespread at the sites at which the short-spurred form is found, extending from the middle of June to early August.

Insect pollinators

Both forms of *E. parviflora* have specialised pollination systems, each being pollinated by a specific insect species.

I collected or inspected a large number of *Cyrtothyrea marginalis* (Cetoniinae; Scarabaeidae) either visiting the short-spurred form of *E. parviflora* or other species (primarily Asteraceae, but also Iridaceae and Hyacinthaceae) in the vicinity of the orchids. A large number of the beetles of both sexes that I observed or collected at a number of sites, bore pollinaria or viscidia of this form of the orchid (Supplementary Table S3). The short open spur of this form accommodates the blunt anterior morphology of the beetles (Figs. 1E and 1F).

In contrast, the long-spurred form appears to be pollinated solely by the solitary bee *Amegilla fallax* (Anthophorinae; Apidae). I collected 81 of these bees of both sex at two sites at either end of Stockville Valley (Supplementary Table S3). Of these, 20 bees bore pollinaria or viscidia of the long-spurred form. These bees have relatively long proboscides which are matched by the long, slender spurs of the long-spurred form (Fig. 1B).

Pollinarium reconfiguration and visit times

I observed a number of visits of *C. marginalis* beetles to inflorescences of the short-spurred form. These visits typically entail beetles alighting on the dense inflorescences, clambering around the inflorescence and entering two or three flowers and depositing or extracting pollinia. Visits by the beetles to the inflorescences lasted 69 seconds on average (n = 5). This is shorter than the average pollinarium reconfiguration time of 119 seconds (Supplementary Fig. S3).

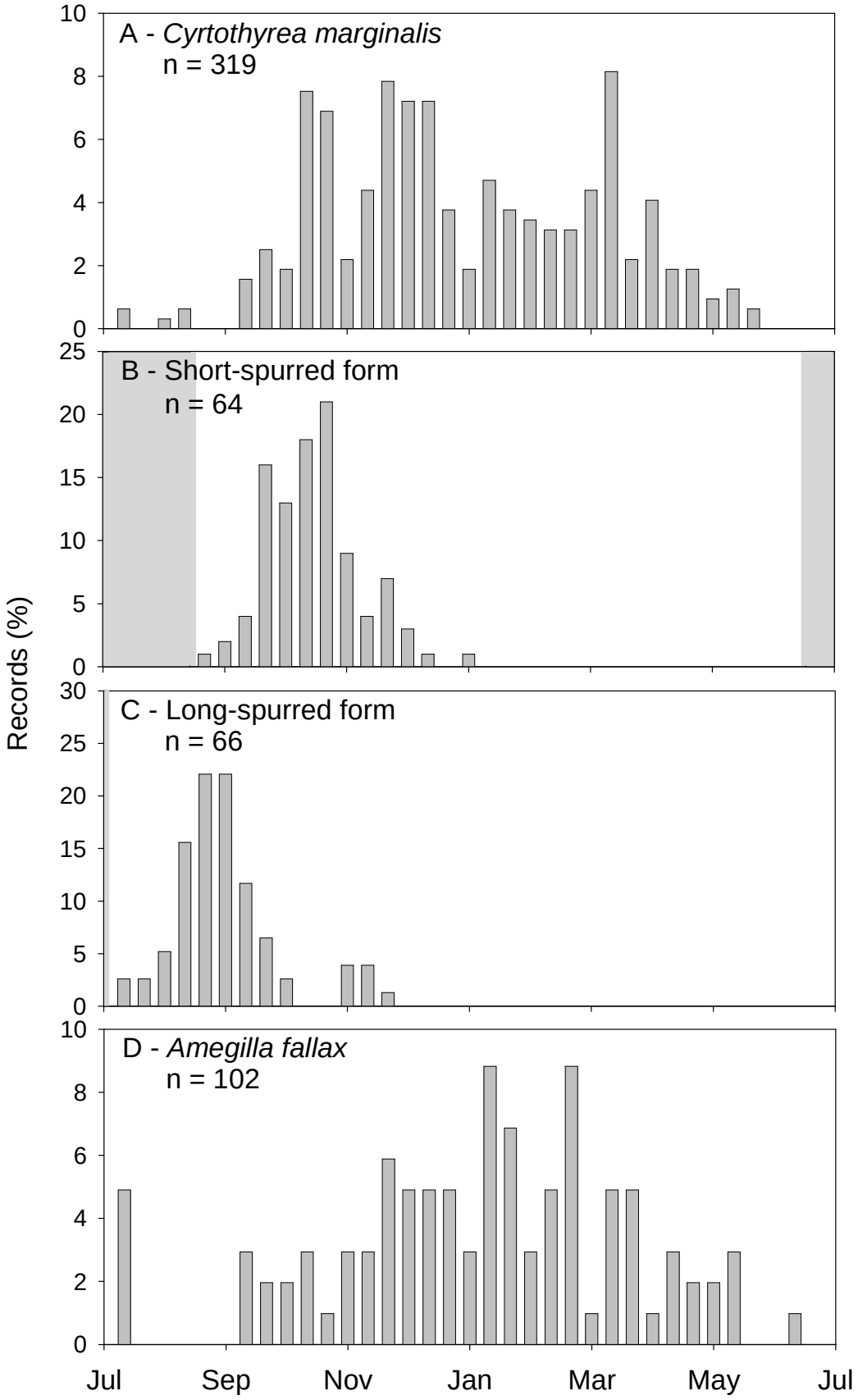

Figure 5: Flowering phenology of the short- (B) and long-spurred (C) forms of *E. parviflora* in relation to the flight periods of *C. marginalis* (A) and *A. fallax* (D), their respective pollinators. The average frost periods for sites where each form is found is indicated in grey.

The majority of the fast moving *A. fallax* bees were collected while foraging for nectar on a variety of other food plants. I did, however, observe and record the duration of two visits (of 25 and 22 seconds) to inflorescences of the long-spurred form. As in the case of the short-spurred form, visits times by *Amegilla* bees to the long-spurred inflorescences (mean 23.5 s) were shorter than the mean pollinarium reconfiguration time of 48 seconds (Supplementary Fig. S3).

Scent analysis

Only the central, rugose ridges of the central lobe of the labellum were stained by neutral red (excluding the stigmatic cavities and pollinia) indicating this as the main site of scent production in these deceptive orchids. The scent of the short-spurred is dominated by aromatic compounds such as benzaldehyde, anisaldehyde, benzyl alcohol, benzyl benzoate, methyl benzoate as well as the terpenoid geraniol (Supplementary Table S4). In contrast the scent of the long spurred form is dominated by various derivatives of the sesquiterpene farnesene, such as farnasal, 2,3 dihydrofarnesal, farnasol, and 2,3 dihydrofarnesol (Supplementary Table S4).

Scent choice experiments.

The *C. marginalis* beetles responded positively to the scent of flowers of the short-spurred form. Significantly more beetles choose the arm of the olfactometer containing the flowers of the short-spurred form over the arm without any scent (Fig. 6B). The beetles show a preference for the scent of the short-spurred form over that of the long spurred form, with significantly more beetles choosing the arm with flowers of the short-spurred form of over the arm containing the scent of the long-spurred form (Fig. 6C).

Visitation rates and pollen transfer efficiency

Rates of pollinaria removal and deposition as well as overall visitation rates are substantially higher in the beetle-pollinated short-spurred form compared to the bee pollinated long-spurred form (Supplementary Table S5). This translates into very high pollen transfer efficiencies in the short-spurred form with nearly a quarter of all removed pollinia being subsequently deposited on stigmas. In contrast, only 6% of removed pollinia are deposited on stigmas of the long-spurred form. Pollination failure (visits that remove the anther cap of the flower, but not the pollinarium) is slightly higher in the beetle-pollinated form.

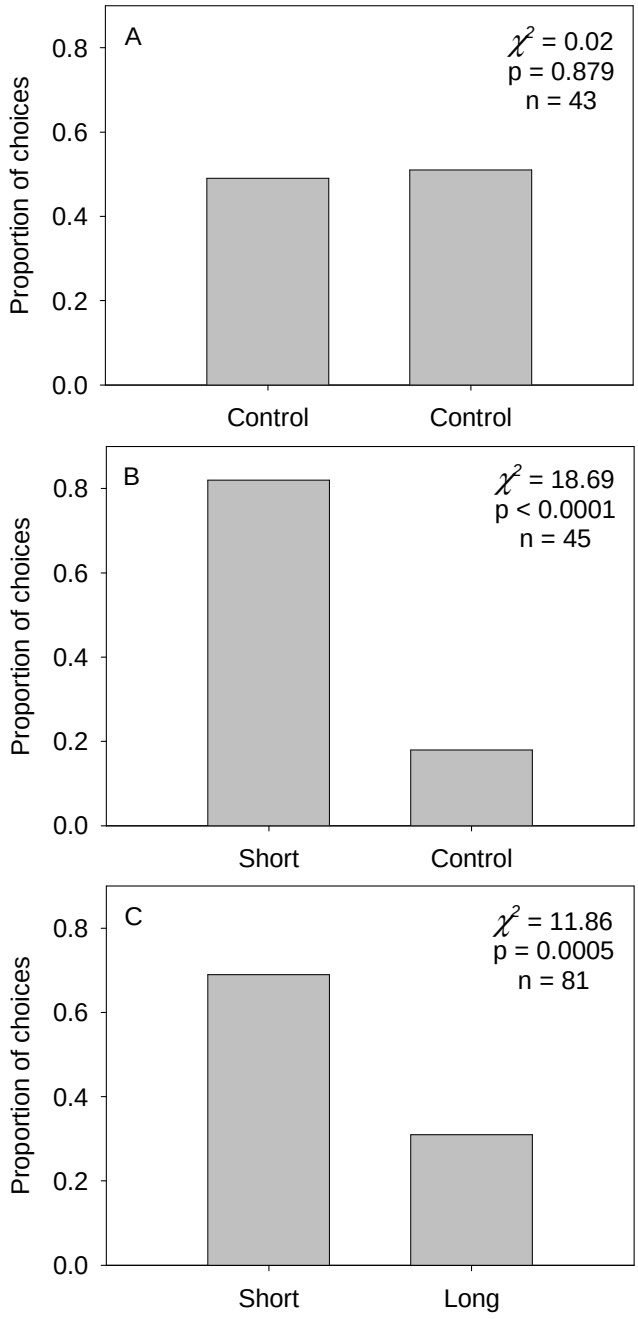

Figure 6A-C: Choices made by *C. marginalis* beetles in a y-maze olfactometer. A) Olfactometer with empty arms aligned correctly to the sun. B) Olfactometer with one arm containing flowers of the short-spurred form of *Eulophia parviflora.* C) Olfactometer with one arm containing flowers of the short-spurred form of *Eulophia parviflora* and the other containing flowers of the long-spurred form.

Translocation experiment

There were no significant differences in rates of pollinaria removal ($U = 162$, $p = 0.85$, $n_1 = 21$, $n_2 = 16$) and pollinia deposition ($U = 152$, $p = 0.62$, $n_1 = 21$, $n_2 = 16$) between inflorescences of the long-spurred form translocated within a natural population and those translocated 60 km outside the natural distribution range.

Spur shortening experiment

Experimental shortening of the spurs of the long-spurred form led to significant reductions in pollinaria removal: only 5 flowers (6%) with shortened spurs had their pollinaria extracted, while 27 (35%) of the control flowers had pollinaria removed ($t = 4.33$, $p < 0.0001$). Pollen deposition on flowers in both treatment groups was too infrequent for statistical analysis.

Breeding system

Both forms of *E. parviflora* are self-compatible and capsules produced by the selfing treatment are comparable in weight to those produced by cross-pollination. In both forms however, the quality of seed produced in the self-pollinated capsules is significantly inferior to seed produced by out-crossing. In the short-spurred plants, selfed capsules produce 50% fewer fertile seeds while in the long-spurred form, selfed capsules produced four times fewer fertile seed (Table 1). Hand-pollinated flowers usually resulted in fruit production, however none of the bagged and unmanipulated flowers, in both forms, set fruit. This indicates that pollinators are essential for capsule production in both forms of *E. parviflora*. The two forms are clearly interfertile with reciprocal crosses both setting fruit and seeds of comparable quality to those produced by cross-pollination within each form.

Table 1: Results of experiments to determine the breeding systems of the two forms of *E. parviflora*.

	Short-spurred form			
	Bagged and unmanipulated	Self-pollinated	Cross-pollinated	Short-spurred[2] x Long-spurred
Percentage fruit set (n)	0 (n = 18)	66 (n = 29)	70 (n = 26)	85 (n = 7)
Mean (± se) capsule & seed mass in grams[1] (n)	-	0.48 ± 0.08 (n = 19)	0.56 ± 0.09 (n = 18)	0.33 ± 0.05 (n = 6)
Mean (± se) percentage of seeds with embryos[1] (n)	-	44.7 ± 6.5[a] (n = 19)	83.8 ± 4.1[b] (n = 18)	86.3 ± 2.5[b] (n = 6)
	Long-spurred form			
	Bagged and unmanipulated	Self-pollinated	Cross-pollinated	Long-spurred[2] x Short-spurred
Percentage fruit set (n)	0 (n = 32)	Not recorded	Not recorded	Not recorded
Mean (± se) capsule & seed mass in grams[1] (n)	-	0.22 ± 0.02 (n = 9)	0.23 ± 0.03 (n = 8)	0.29 ± 0.05 (n = 7)
Mean (± se) percentage of seeds with embryos[1] (n)	-	20.2 ± 5.3[a] (n = 9)	86.6 ± 1.6[b] (n = 8)	84.5 ± 1.6[b] (n = 7)

1 Percentage data were arcsine transformed and compared using ANOVA. Homogenous groups were determined using a post hoc Tukeys test.

2 Pod parent.

DISCUSSION

The results of this study are consistent with the specific predictions generated by the hypothesis that floral divergence in the *Eulophia parviflora* complex reflects an evolutionary shift between different pollinators. Specifically, these analyses show that there are two forms in the complex, each with a distinctive suite of correlated traits that includes flower shape (Fig. 3), scent (Supplementary Table S4), phenology (Fig. 5) and pollinarium reconfiguration time (Supplementary Fig. S3). These observations at a wide range of sites show that each of these forms is pollinated by a specific insect species (a bee and beetle species, respectively). Experiments (spur manipulation, scent choices), phenological matching of flowering and pollinator emergence, and correlations between pollinator visitation times and pollinarium reconfiguration, suggest a functional role for the traits that characterize these forms.

This study is unusual in that a whole suite of traits was considered, unlike many previous studies which focused on pollinator-driven evolution of single traits, such a spur length (Robertson & Wyatt 1990, Johnson 1997, Johnson & Steiner 1997, Boyd 2004), scent chemistry (Pellmyr 1986, Johnson *et al.* 2005) and flowering phenology (Herrera *et al.* 2002). Interestingly, I found no quantitative difference between the flower colours of the two forms of *E. parviflora* which could indicate that scent is the primary form of advertising used to attract pollinators (Supplementary Fig. S2).

These olfactometer experiments showed that beetles were strongly attracted to the fruity, cherry-like scent of the short-spurred form, and that they preferred the scent emitted by flowers of this form over the scent emitted by a similar number of flowers of the long-spurred form (Fig. 6C). A number of the constituents of the scent of this form have been shown to be attractive to various beetles including other cetoniid beetles. These include geraniol (Klein & Edwards 1989, Cherry & Klein 1992, Imai *et al.* 1998, Toth *et al.* 2003), benzaldehyde (Leal *et al.* 1994), benzyl alcohol (Leal *et al.* 1994), anis aldehyde (Imai *et al.* 2002), (E) ocimene, methyl benzoate (Leal *et al.* 1996, Jürgens *et al.* 2000, Hammack & Petroski 2004), benzyl benzoate (Leal *et al.* 1994) and linalool (Donaldson *et al.* 1990, Imai *et al.* 1998, Johnson *et al.* 2007), although there is evidence of species specific responses to specific compounds and scent mixtures (Leal 1998). Further experiments are required to unravel the relative attractivness of the different compounds making up the scent the long-spurred form. Compounds such as eugenol and nerolidol were shown by Donaldson *et al.*

(1986, 1990) to be highly attractive to a number of South African cetoniids (and rutelinids), however these are found at very low concentrations in the scent of the short-spurred form.

There are relatively few reports of beetle-pollination in orchids, but in most of these cases the flowers are reported to be scented and pale green or brown in colour (Nilsson 1981, Singer & Cocucci 1997, Peter & Johnson 2006a, Johnson *et al.* 2007). The pollination system of the short-spurred form of *E. parviflora* has similarities with that of *Pteroglossaspis ruwenzoriensis* which is also pollinated by cetoniid beetles and have dense inflorescences of scented flowers and petals with dark adaxial surfaces (Singer & Cocucci 1997). There are notable differences however and *P. ruwenzoriensis* has drab pale pink and green inflorescences; is rewarding, producing "jelly-like" nectar; and has a yeast-like scent. The beetle-pollinated short-spurred form also has similarity with *Ceratandra grandiflora*, a yellow-flowered orchid with dense inflorescences. Monkey beetles aggregate on the deceptive inflorescences which serve as mating rendezvous sites (Steiner 1998). Crowded inflorescences appear to be a key characteristic of beetle-pollinated orchids and are consistent with the general syndrome of beetle pollination (cf. van der Pijl 1961, Faegri & van der Pijl 1979, Bernhardt 2000). Crowded inflorescences are an efficient way of producing large attractive displays from small flowers, and also allow beetles to clamber over the inflorescences.

Only bees (both sexes of *A. fallax*) were seen to visit flowers of the long-spurred form of *E. parviflora*. Bees exhibit a typical zig-zagging flight pattern when approaching inflorescences, suggesting that scent is an important component of the attractiveness of the flowers. Three of these approaches ended with the bees briefly hovering close to the inflorescence before flying away, while two approaches ended with the bees landing and probing a flower. The scent of the long-spurred form is strongly dominated by various derivatives of the terpenoid farnesene (Supplementary Table S4), compounds know from a number of floral scents including various orchid species (Knudsen *et al.* 2006). The isomers of farnesol appear to be important components of the female attracting pheromone in *Xylocopa* carpenter bees (Williams *et al.* 1987, Minckley *et al.* 1991) and *Bombus pratorum* (Bergman & Bergström 1997). *Xylocopa* bees have a "dispersed lek system", with male scent marking a territory to attract female bees for mating (Andersen *et al.* 1988). Male *Bombus* bees have similar scent mark behaviour to attract virgin queens (Bergman & Bergström 1997).

It is possible therefore that farnesene derivatives play an important role in the biology of *A. fallax*, possibly being used to mark communal lek positions or to coordinate mating

rendezvous. If this is the case this may represent a novel aspect of sexual deception that has not been documented previously. Johnson *et al.* (2005) speculated that the attraction of both male and female *Tetraloniella* (Anthophorinae) bees, known to have a strong lekking behaviour (Eardley pers comm.), to the deceptive flowers of *Disa spathulata* may be due to mimicry of scent blends used by bees to mark nest sites or leks. However, the biological role of floral scent compounds in attraction of bees to these orchids has still to be confirmed using behavioural assays with single compounds and blends. Interestingly, farnesyl hexanoate acts as a repellent compound following pollination in the sexually deceptive *Ophrys sphegodes* (Schiestl & Ayasse 2001) mimicking the post mating cues of the female bees which serve as models (Schiestl & Ayasse 2000).

There are several other uncertainties associated with this study that would require further study to resolve. Firstly, I was unable to confirm the basis for the geographical structure of forms within the complex - the long-spurred form is found at low altitude along the coast, while the short-spurred form is mainly found at high altitudes (Fig. 2) - as the prediction that pollination success of forms should be higher within its own distribution range was not upheld by a reciprocal translocation experiment. In addition, both pollinator species have wide current distributions in southern Africa (Holm & Marais 1992, Eardley 1994). Thus it is not clear whether the geographical structure of the forms reflects geographical availability of pollinators, as predicted by the Grant-Stebbins model.

A comparison of flowering and phenology times of respective plant and pollinator as well as local frost periods (Fig. 5) suggests a possible alternative explanation for the distribution of the two forms. The flowering of the inland short-spurred form peaks in October coinciding with the emergence of the *Cyrtothyrea marginalis* beetles. For most sites, this is at least a month after the end of the winter frosts. In contrast, the long-spurred form begins flowering at the end of the austral winter in July, coinciding with the very early emergence of the *Amegilla fallax* bees. This early flowering to exploit newly emerged bees is possible at the coast because of the lack of frost, but may not be viable at colder inland localities.

The second uncertainty relates to the direction of the shift (cf. Whittall & Hodges 2007). There is no species-level phylogeny available for *Eulophia* that would allow us to optimize the likely pollination system of the immediate ancestor of the two forms and thereby establish whether the shift was bee to beetle, beetle to bee or from a third pollination system in the immediate ancestor to beetle and bee in these daughter forms. To date only bee and beetle

pollination has been documented in this genus so there is little support for this third option. Most of the related species have relatively long and slender floral spurs and lax inflorescence morphologies and few flowers open at one time (cf. Peter & Johnson 2008 [chapter 2], chapter 7), from which I can infer that the shift was from bee to beetle pollination, but this remains speculative without a well-resolved phylogeny. This would entail a shift from long to short spurs and be at odds with the general evolutionary trend for shifts from shorter to longer spurs that was demonstrated by Whittall & Hodges (2007).

The third uncertainty relates to whether the forms should be considered ecotypes or fully developed species. The crossing experiments suggest that the two forms are interfertile (at least capable of forming hybrid seeds with embryos). The two forms also coexist at some sites, with differences in flowering time and pollinators serving as the main isolating barriers. This ability to "withstand the challenge of sympatry" (Coyne & Orr 2004) leads us to conclude that the forms are indeed good biological species with ethological and phenological isolating barriers. This is at odds with the current taxonomy, but would not be the first case where biological species have been overlooked by taxonomists working mainly from herbarium specimens. Sites where the two forms coexist also conveniently substitute for a common garden experiment in establishing that the differences between the two forms have a genetic basis and are not the result of phenotypic plasticity.

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[Chapter 6 – supplementary material]

Supplementary Table S1: Mean character states for the two forms. The characters listed here are those used in the phenetic analysis.

	Character	Mean Long-spurred form	Mean Short-spurred form	t	df	p
1	Renumbered date	54.5	89.9	-10.0	84	<0.0000
2	Height of Scape (mm)	244.2	437.3	-10.3	89	0.0000
3	Height of vegetative shoot at anthesis (mm)	44.8	128.3	-6.6	88	0.0000
4	Leaves clasping central axis, Yes (1), No (0)					
5	Diameter of roots within 10mm of tuber (mm)	4.3	4.8	-4.5	79	0.0000
6	Average diameter of tuber (mm)	19.2	26.5	-8.2	78	0.0000
7	Maximum width of scape at ground level (mm)	2.8	5.0	-7.0	90	0.0000
8	Scape cross-section, 0val (0), Round (1)					
9	Number of nodes above ground (scape below flowers)	2.5	4.3	-11.4	91	0.0000
10	Number of nodes above ground (with flowers)	6.1	15.7	-16.7	91	0.0000
11	Average internode length of scape (below flowers) (mm)	55.9	76.2	-5.4	91	0.0000
12	Average internode length flowering portion of inflorescence (with flowers) (mm)	16.3	9.6	5.1	91	0.0000
13	Length of sheaths (mm)	28.0	40.1	-5.9	91	0.0000
14	Length of bracts (mm)	13.2	14.7	-2.4	91	0.0176
15	Distance from bottom most flower to top of the scape (mm)	67.3	90.5	-4.1	91	0.0001
16	Inflorescence dense/lax (length flowering portion / number of flower nodes)	11.0	5.9	9.2	91	0.0000
17	Flower resupinating, Yes (1), No (0)					
18	Length of pedicel (open flowers) (mm)	19.2	12.7	9.6	91	0.0000
19	Width of pedicel at point of attachment (mm)	2.1	3.5	-3.1	91	0.0024
20	Length of pedicel / width of pedicel	9.3	4.6	14.6	91	0.0000
21	Length of dorsal sepal (mm)	15.3	12.6	6.3	90	0.0000
22	Breadth of dorsal sepal (mm)	5.8	7.2	-6.5	90	0.0000
23	Length dorsal sepal / breadth dorsal sepal	2.7	1.8	12.0	90	0.0000
24	Depth of dorsal sepal (mm)	1.0	1.2	-2.0	91	0.0537
25	Thickness of dorsal sepal (mm)	0.4	0.5	-6.4	91	0.0000
26	Length of lateral sepal (mm)	16.1	12.5	9.3	91	0.0000
27	Breadth of lateral sepal (mm)	5.9	7.4	-6.6	91	0.0000
28	Length lateral sepal / breadth dorsal sepal	2.8	1.7	15.4	91	0.0000
29	Depth of lateral sepal (mm)	1.9	2.1	-0.8	91	0.4359
30	Thickness of lateral sepal (mm)	0.4	0.5	-3.9	91	0.0002
31	Length of petal (mm)	13.1	10.4	10.2	91	0.0000
32	Breadth of petal (mm)	7.4	6.9	2.2	91	0.0281
33	Length of petal / breadth of petal	1.8	1.5	5.6	91	0.0000
34	Depth of petal (mm)	1.5	1.3	1.6	91	0.1057
35	Thickness of petal (mm)	0.3	0.3	-0.1	91	0.9192
36	Length of column to tip of anther cap (mm)	7.6	6.3	7.8	87	0.0000
37	Length of column with anther cap removed (mm)	6.5	5.6	6.2	91	0.0000
38	Width of column at widest point (behind anther cap) (mm)	3.7	4.3	-5.5	91	0.0000
39	Length of column / width of column	2.1	1.5	11.6	87	0.0000
40	Length of stipe (mm)	1.2	1.4	-3.3	85	0.0013
41	Width of stipe (mm)	0.7	1.1	-9.4	85	0.0000
42	Length of stipe / width of stipe	1.7	1.3	5.8	84	0.0000
43	Length of pollinium (mm)	0.9	1.0	-2.0	86	0.0463
44	Width of pollinium (mm)	0.7	0.8	-2.7	86	0.0091
45	Length of pollinium / width of pollinium	1.4	1.3	0.5	86	0.6172

46	Distance from Base of column (at receptacle) to the basal edge of the stigmatic cavity (mm)	5.0	3.7	8.8	91	0.0000
47	Length of stigmatic cavity (mm)	1.2	0.9	5.9	91	0.0000
48	Width of stigmatic cavity (mm)	2.3	2.8	-6.5	91	0.0000
49	Depth of column at the tip with anther cap removed (mm)	1.9	2.5	-6.2	90	0.0000
50	Length of mentum (midline of petal to point of attachment with labellum) (mm)	3.5	2.9	4.6	90	0.0000
51	Width of labellum at point of attachment to mentum (mm)	3.1	3.5	-2.8	91	0.0064
52	Length of labellum - attachment to tip of central lobe (mm)	12.3	9.7	9.9	91	0.0000
53	Length of labellum / width of labellum	2.1	2.0	0.3	91	0.7488
54	Length of side lobe (mm)	6.8	4.8	10.3	91	0.0000
55	Width of labellum central lobe (mm)	6.2	5.1	4.3	91	0.0000
56	Depth of labellum (mm)	2.2	1.9	2.8	91	0.0067
57	Thickness of labellum (mm)	0.6	0.5	1.9	89	0.0624
58	Length of spur (tip to mentum attachment) (mm)	7.0	3.5	20.7	91	0.0000
59	Length of spur (tip to midlobe - side lobe junction) (mm)	11.3	6.1	17.3	91	0.0000
60	Width of spur at tip (mm)	1.8	2.1	-2.0	91	0.0517
61	Outside width of spur below receptacle (mm)	4.0	4.5	-3.7	91	0.0004
62	Inside width of spur at entrance (mm)	2.3	2.8	-3.6	91	0.0005
63	Maximum separation between labellum & anther cap (mm)	0.8	1.8	-10.9	90	0.0000
64	Distance between tip of labellum & tip of dorsal sepal (mm)	23.5	13.1	10.5	90	0.0000
65	Distance between tip of labellum & tip of lateral sepal (mm)	21.7	9.4	13.9	90	0.0000
66	Distance between tip of labellum and tip of petal (mm)	14.1	5.2	11.0	89	0.0000
67	Distance btwn tip of dorsal sepal & tip of lateral sepal (mm)	23.0	15.2	8.5	90	0.0000
68	Distance from tip of labellum to tip of spur (mm)	15.1	10.2	15.4	90	0.0000
69	Angle between inflorescence axis and column axis (degrees)	96.8	25.7	17.2	90	0.0000
70	Angle between column axis and petal (degrees)	42.8	6.3	9.0	91	0.0000
71	Angle between column axis and lateral sepal (degrees)	87.4	30.1	13.0	91	0.0000
72	Angle between column axis and dorsal sepal (degrees)	88.2	32.0	12.0	91	0.0000
73	Colour of 01 sepal adaxial tip LM opponent	0.5	0.5	-0.4	91	0.7038
74	Colour of 01 sepal adaxial tip MS opponent	0.3	0.2	3.1	91	0.0029
75	Colour of 05 petal adaxial tip LM opponent	0.6	0.5	1.0	91	0.3149
76	Colour of 05 petal adaxial tip MS opponent	0.2	0.2	-1.5	91	0.1290
77	Colour of 06 petal adaxial base LM opponent	0.4	0.4	1.5	88	0.1373
78	Colour of 06 petal adaxial base MS opponent	0.3	0.3	0.8	88	0.3995
79	Colour of 07 petal abaxial tip LM opponent	0.3	0.4	-3.6	91	0.0006
80	Colour of 07 petal abaxial tip MS opponent	0.3	0.3	0.1	91	0.9557
81	Colour of 08 petal abaxial base LM opponent	0.4	0.4	-0.8	91	0.4156
82	Colour of 08 petal abaxial base MS opponent	0.4	0.4	4.1	91	0.0001
83	Colour of 09 labellum adaxial LM opponent	0.4	0.4	-0.5	91	0.6325
84	Colour of 09 labellum adaxial MS opponent	0.4	0.4	-1.7	91	0.0837

Supplementary Table S2: Eigenvalues, percentage variance explained and cumulative variance explained for the PCA analysis.

	Eigenvalue	Percent	Cumulative		Eigenvalue	Percent	Cumulative
1	28.13	33.49	33.49	43	0.18	0.21	98.30
2	9.86	11.74	45.23	44	0.16	0.19	98.49
3	4.66	5.55	50.77	45	0.15	0.18	98.67
4	4.11	4.89	55.67	46	0.14	0.17	98.84
5	2.76	3.28	58.95	47	0.13	0.16	99.00
6	2.72	3.24	62.19	48	0.13	0.15	99.15
7	2.36	2.81	65.00	49	0.12	0.14	99.29
8	2.20	2.61	67.62	50	0.10	0.12	99.41
9	1.89	2.25	69.87	51	0.10	0.12	99.53
10	1.85	2.20	72.07	52	0.09	0.11	99.64
11	1.60	1.90	73.98	53	0.09	0.11	99.75
12	1.37	1.63	75.61	54	0.08	0.10	99.84
13	1.29	1.54	77.15	55	0.07	0.08	99.92
14	1.25	1.49	78.64	56	0.06	0.08	100.00
15	1.23	1.46	80.10	57	0.06	0.07	> 100%
16	1.12	1.33	81.44	58	0.05	0.06	> 100%
17	1.09	1.30	82.74	59	0.05	0.06	> 100%
18	1.06	1.26	84.00	60	0.04	0.05	> 100%
19	0.96	1.14	85.14	61	0.04	0.04	> 100%
20	0.95	1.13	86.27	62	0.04	0.04	> 100%
21	0.82	0.98	87.25	63	0.03	0.04	> 100%
22	0.77	0.92	88.17	64	0.03	0.03	> 100%
23	0.72	0.85	89.02	65	0.02	0.03	> 100%
24	0.66	0.79	89.81	66	0.02	0.02	> 100%
25	0.66	0.78	90.59	67	0.02	0.02	> 100%
26	0.63	0.76	91.35	68	0.01	0.01	> 100%
27	0.56	0.67	92.02	69	0.01	0.01	> 100%
28	0.53	0.63	92.64	70	0.01	0.01	> 100%
29	0.49	0.58	93.22	71	0.01	0.01	> 100%
30	0.47	0.55	93.78	72	0.00	0.00	> 100%
31	0.43	0.51	94.29	73	0.00	0.00	> 100%
32	0.42	0.49	94.78	74	0.00	0.00	> 100%
33	0.37	0.44	95.22	75	0.00	0.00	> 100%
34	0.34	0.41	95.63	76	0.00	0.00	> 100%
35	0.33	0.39	96.02	77	0.00	0.00	> 100%
36	0.30	0.36	96.38	78	-0.01	-0.01	> 100%
37	0.28	0.34	96.72	79	-0.01	-0.01	> 100%
38	0.26	0.30	97.02	80	-0.01	-0.02	> 100%
39	0.25	0.30	97.32	81	-0.02	-0.02	> 100%
40	0.24	0.28	97.60	82	-0.04	-0.05	> 100%
41	0.22	0.26	97.86	83	-0.08	-0.10	> 100%
42	0.19	0.22	98.09	84	-0.25	-0.30	100

Supplementary Table S3: Pollinators collected visiting or bearing pollinaria of the two forms of *E. parviflora*. Totals are given in bold type.

Pollinator	Site[1]	Number of insects observed or collected[2]	Number of insects with intact pollinaria[2] (%)[3]	Number of insects with only viscidia[2] (%)[3]	Number of insects approaching or visiting[4] (%)[3]
Long-spurred form					
Amegilla fallax	1a	8	0 (0)	0 (0)	0 (0)
Amegilla fallax	1b	18	0 (0)	8 (44)	6 (33)
Amegilla fallax	1c	63	4 (6)	8 (13)	1 (2)
		89	**4 (4)**	**16 (18)**	**7 (9)**
Short-spurred form					
Cyrtothyrea marginalis	1a	519	30 (6)	3 (1)	7 (1)
Cyrtothyrea marginalis	2a	15	5 (33)	2 (13)	0 (0)
Cyrtothyrea marginalis	3	8	1 (13)	0 (0)	0 (0)
Cyrtothyrea marginalis	4	34	13 (38)	4 (12)	1 (3)
Cyrtothyrea marginalis	7	4	0 (0)	1 (25)	1 (25)
Cyrtothyrea marginalis	8	9	2 (22)	0 (0)	0 (0)
		589	**51 (9)**	**10 (2)**	**9 (2)**

[1] Sites number according to Fig. 2 and include in KwaZulu-Natal: (1a) Krantzkloof Nature Reserve, Kloof, Durban; (1b) Top of Stockville Valley, Kloof, Durban; (1c) Bottom of Stockville Valley, Kloof, Durban; (2a) Victoria Country Club, Pietermaritzburg; (3) Umgeni Valley Nature Reserve, Howick; (4) Road verge near Balgowan in the KwaZulu-Natal midlands. Sites in the Eastern Cape: (7) Outskirts of the town of Maclear; (8) Rietberg, Grahamstown.

[2] Either on the inflorescences of the study species or visiting other plants in the vicinity.

[3] Percentage of insects at the specific site, or (in bold) percentage of insects from all sites.

[4] Visiting or approaching the inflorescences of the study species.

Supplementary Table S4: Floral scent composition for three plants of each of the two forms of *Eulophia parviflora*.

	Long			Short		
	Krantzkloof 14 Aug 2001	Krantzkloof 13-Nov-2000	Stockville Valley 24-Oct-2002	Umgeni Valley 24-Oct-2002	Maclear 20-Nov-2000	Krantzkloof 09-Oct-2000
ALIPHATICS						
Ethyl palmitate long chain aliphatic	-	-	-	-	0.3	-
Acids						
Nonanoic acid	**1.8**	-	-	-	-	0.10
Capric acid (= decanoic acid)	-	-	-	-	-	0.1
Caprylic acid (= octanoic acid)	-	-	-	-	-	0.05
Alkenes						
1-Pentadecene	-	-	-	-	-	0.90
Pentadecadiene [(Z)-1,8-Pentadecadiene]	-	-	-	-	-	0.10
Aldehydes						
Heptanal	0.3	-	-	-	-	-
Decanal	**1.7**	**1**	**1**	**2.1**	0.3	0.2
Hexanal	0.2	-	-	-	-	-
Nonanal	**2.6**	0.4	**1**	**8.2**	0.3	0.2
Octanal	0.4	-	-	**1**	-	0.08
Tetradecanal	-	-	-	-	-	0.1
Alcohols						
Octanol	0.4	-	-	-	-	-
Nonanol	0.2	-	-	-	-	0.06
Hexanol	-	0.8	-	-	-	0.2
(Z) - 3 - Hexanol	-	**2.5**	-	-	-	0.06
1-Octen-3-ol	-	-	-	-	-	0.1
Decanol	-	-	-	-	-	0.07
(Z)-3-Nonen-1-ol	-	-	-	-	-	0.1
Esters						
Methyl 2-ethylcaproate	-	-	-	-	-	0.06
BENZENOIDS						
Aldehydes						
Benzaldehyde	0.1	-	-	**4**	**2.5**	**8.9**
Anis aldehyde	-	-	-	**16.5**	**28**	**3.3**
Alcohols						
Benzyl alcohol	-	-	-	**3.6**	**0.3**	**1.5**
2-Phenylethanol (phenylethyl alcohol)	-	-	-	**1.8**	**1**	**2**
Anisyl alcohol	-	-	-	-	0.4	-
Para-methyl anisole (= p-cresol)	-	-	-	-	-	0.2
Esters						
Benzyl benzoate	-	-	-	**1.6**	**2.5**	**-**
Methyl benzoate	-	-	-	**14**	**2.7**	**9.8**
Benzyl-3-methylbutanoate (benzyl isovalerate)	-	-	-	-	-	0.07
Methyl anisate (= Methyl methoxybenzoate)	-	-	-	0.2	-	-
Ethers						

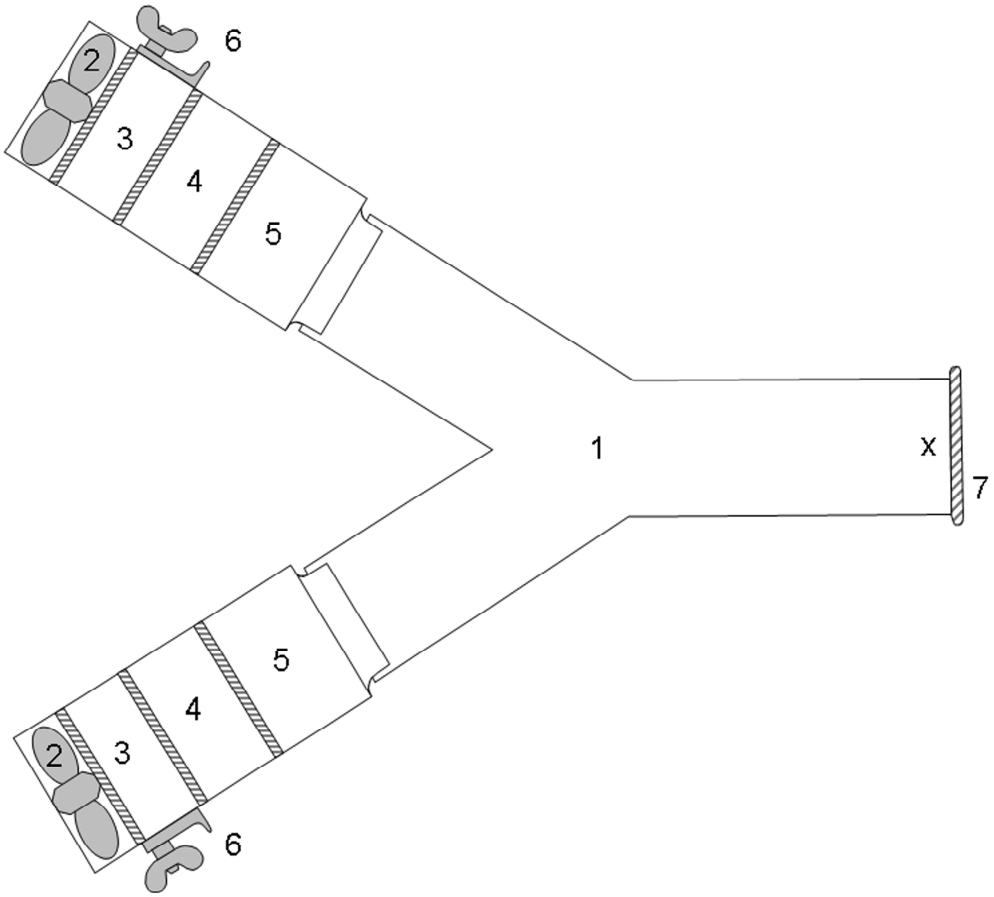
Para-methoxybenzyl methyl ether	-	-	-	0.2	**1.2**	-
1,4_Dimethoxybenzene (hydroquinone, dimethyl ether)	-	-	-	-	**6**	**2.6**
N-group						
Phenylacetonitrile (= benzyl nitrile)	-	-	-	-	**2**	-
PHENYLPROPANOIDS						
Alcohols						
Eugenol	-	-	-	-	0.8	0.2
Esters						
Methyl (E) cinnamate	**19**	-	-	-	-	0.1
Methyl (Z) cinnamate	**5.3**	-	-	-	-	-
ISOPRENOIDS						
Irregular terpenes						
6-Methyl-5-hepten-2-one	**3**	-	**10**	**1.2**	-	0.4
(E) - 4,8 - Dimethyl - 1,3,7 - Nonatriene	-	-	-	**1.6**	-	0.4
(E,E) - 4,8,12 - Trimethyl - 1,3,7,11 - tridecatetraene	-	-	-	0.8	-	-
Monoterpenes						
Geraniol	-	-	-	0.9	-	**29.9**
Geranial	-	-	-	-	-	**9.6**
Methyl geranate	-	-	-	-	-	**1**
Geranyl acetate	-	-	-	-	-	0.06
Geranyl formate	-	-	-	-	-	0.1
2,3-Epoxi geraniol	-	-	-	-	-	0.3
6,7-Epoxi geraniol	-	-	-	-	-	0.3
Geranic acid	-	-	-	-	-	0.1
Linalool	-	-	0.9	**2.4**	-	**6.5**
Citronellol	-	-	-	0.6	-	0.1
Neral	-	-	-	-	-	**2.2**
Nerol monoterpene	-	-	-	-	-	**1.5**
(6)7-epoxyneral	-	-	-	-	-	0.05
alpha - pinene	0.2	-	-	-	-	-
beta- - pinene	0.2	-	-	-	-	-
(Z) Ocimene	-	-	-	0.6	-	-
(E) Ocimene	-	-	-	**10**	**1.2**	0.5
Myrcene	-	-	-	-	-	0.4
Sabinene	-	-	-	-	-	0.1
Limonene	-	-	-	-	-	0.1
trans-Linalool oxide (furanoid)	-	-	-	-	-	0.1
cis-Linalool oxide (furanoid)	-	-	-	-	-	0.1
2,6-Dimethyl-3,7-octadien-2,6-diol	-	-	-	-	-	0.1
Sesquiterpenes						
(Z,E) - Farnesal	0.8	-	-	-	-	-
(E,E) - Farnesal	**1.2**	**3.8**	**2.6**	-	-	-
(E,E) - Farnesol	**-**	**23**	**8.6**	-	-	-
(E) - 2 (3) - Dihydrofarnesol	**31**	**24**	**20.6**	-	-	-
(E) - 2 (3) - Dihydrofarnesal	**2.2**	**4.5**	**15**	-	-	-
(E) - Nerolidol	0.2	-	-	-	-	0.5

AMINES & OTHER NITROGEN-CONTAINING COMPOUNDS						
Indole	0.2	-	-	-	-	0.2
I-Nitro-2-phenylethane	-	-	-	-	0.4	-
MISCELLANEOUS CYCLIC COMPOUND						
2,3-Dimethyl-2,4-nonadien-4-olide	-	-	-	-	-	0.1
Percent	**72**	**60**	**59.7**	**71.3**	**50.3**	**86.4**

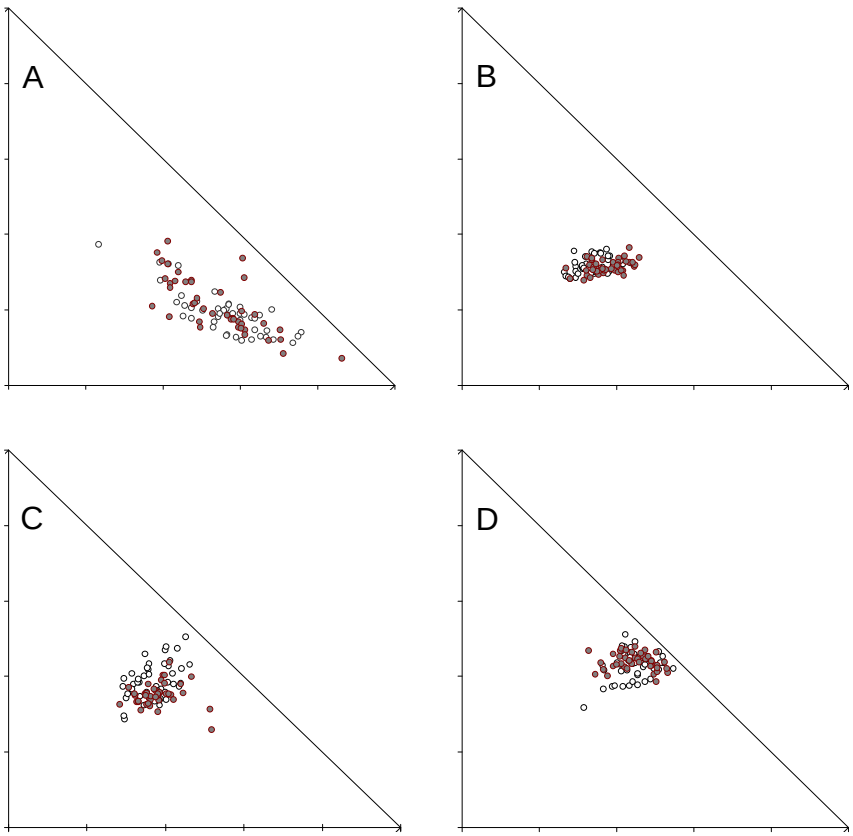
Supplementary Table S5: Pollen transfer efficiencies in different populations of the two forms of *E. parviflora*.

Date	Locality[1]	n - inflorescences	n - flowers	% flowers with removal	% flowers pollinated	% Visited	% Failure	PTE %
Long-spurred form								
12 Aug 2001	Krantzkloof (1a)	28	102	49.0	8.8	54.9	7.1	9.0
13 Aug 2001	Krantzkloof (1a)	36	174	17.2	3.4	21.3	16.2	10.0
19 Aug 2001	Stockville Valley (1c)	24	97	11.3	0.0	14.4	21.4	0.0
24 Aug 2001	Krantzkloof (1a)	7	31	35.5	6.5	38.7	8.3	9.1
06 Aug 2002	Umtamvuna (6)	6	28	10.7	0.0	10.7	0.0	0.0
10 Aug 2002	Stockville Valley (1c)	26	117	12.8	2.6	13.7	0.0	13.3
12 Aug 2002	Stockville Valley (1c)	34	213	9.9	1.4	11.7	16.0	7.1
19 Aug 2002	Stockville Valley (1c)	13	55	9.1	1.8	9.1	0.0	10.0
27 Aug 2002	VCC translocation (2)	22	101	18.8	2.0	21.8	9.1	10.5
27 Aug 2002	Stockville Valley (1c)	19	94	13.8	0.0	17.0	18.8	0.0
16 Sep 2002	Krantzkloof (1a)	8	28	14.3	0.0	17.9	20.0	0.0
	Average			***18.4***	***2.4***	***21.0***	***10.6***	***6.3***
Short-spurred form								
13 Oct 2000	VCC (2)	27	262	69.5	30.5	73.3	n/r	22.0
16 Oct 2000	Balgowan (4)	5	50	56.0	16.0	60.0	n/r	14.3
23 Oct 2000	Umgeni Valley (3)	4	36	61.1	8.3	61.1	n/r	6.8
16 Sep 2002	Krantzkloof (1a)	7	43	55.8	20.9	58.1	4.0	37.5
02 Oct 2002	VCC (2)	9	65	44.6	21.5	46.2	3.3	36.2
16 Oct 2002	VCC (2)	24	138	52.2	18.8	57.2	7.6	20.1
08 Nov 2006	Grahamstown[2] (8)	98	98	14.3	5.1	23.5	34.8	32.1
08 Nov 2006	Grahamstown[3] (8)	11	70	57.1	7.1	68.6	14.6	10.0
	Average			***51.3***	***16.1***	***56.0***	***12.9***	***22.4***

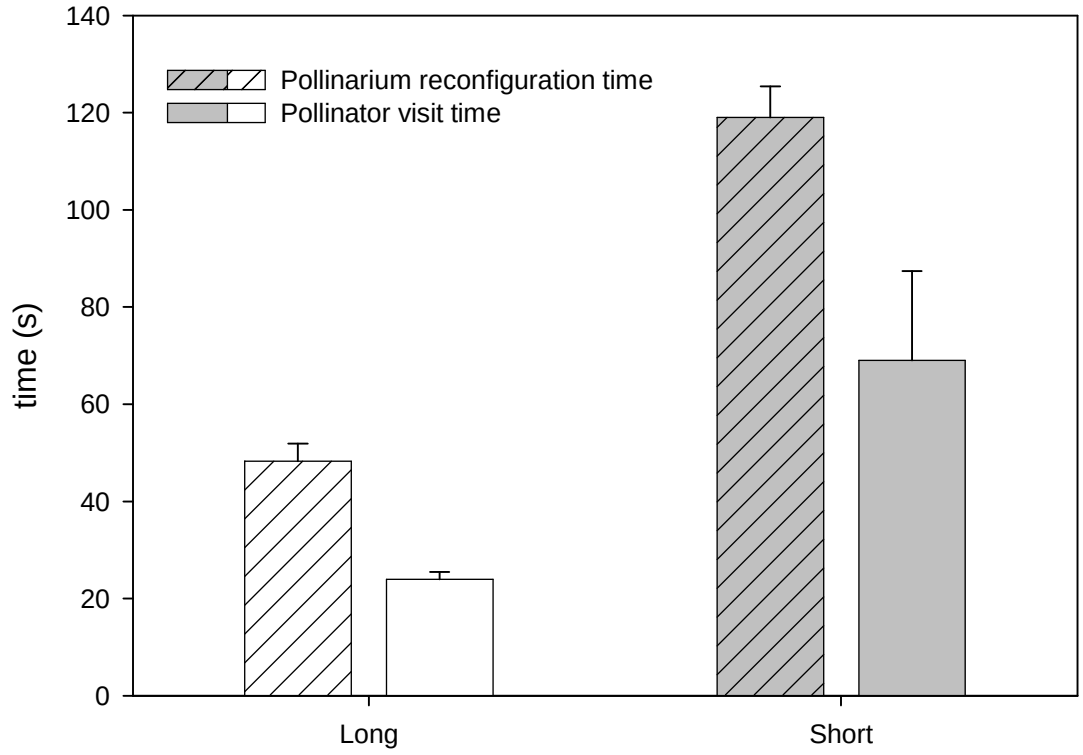
Values in bold indicate the average for the two forms.
[1] Sites numbered according to Fig. 2.
[2] Large dense population.
[3] Small sparse population.



Supplementary Figure S1: Beetle scent preferences were determined using a Perspex Y-shaped olfactometer (1). Small computer (2) fans were used to blow ambient air through a balancing chamber (3), the scent chamber (4) housing the scent sample and the end holding cage (5). Parts 2 to 5 are formed by a stainless steel housing with each chamber separated by stainless steel mesh (▨). Flow rates in each of the two arms were balanced using a bleed system (6). Insects were introduced at point "x" and their choices noted. A stainless steel mesh screen (7) prevented insects exiting the choice maze.



Supplementary Figure S2: Colours of various parts of the flowers of the long- (○) and short-spurred (●) forms, summarised according to the Endler segment classification method. A) Adaxial surface of the lateral petal tip, B) abaxial surface of the lateral petal base, C) abaxial surface of the lateral petal tip and D) adaxial surface of the labellum central lobe.



Supplementary Figure S3: Mean pollinaria reconfiguration is longer than mean pollinator visit time to inflorescences in both forms. Error bars represent the standard error of the mean.

Pollination case histories

[chapter 7]

Bee and Beetle pollination in the South African species of *Eulophia*

ABSTRACT Prior to the current research, little has been known about the pollination biology of the large (c. 230 species) African genus *Eulophia*. This chapter reports the discovery of pollination by bees in five species and beetles in two species, and includes analyses of floral spectral reflectance, post-removal pollinarium reconfiguration, pollen transfer efficiency and breeding systems of these species. Several *Eulophia* species with large showy flowers arranged in racemes were found to be pollinated by various species of *Xylocopa* (Anthophorinae, Apidae) or *Megachile* (Megachilidae), while species of *Eulophia* with smaller flowers in racemes were found to be pollinated by smaller anthophorid (Anthophorinae, Apidae) or halictid (Halictidae) bees. In contrast, *Eulophia* species with congested, capitate inflorescences were found to be pollinated by beetles, primarily various flower chaffers (Cetoniinae; Scarabaeidae) and, in one species, click beetles (Elatyridae; [chapter 3]).
All seven taxa examined possess one of three modes of pollinarium reconfiguration (two modes of pollinarium bending and one mode of anther cap retention) that are likely adaptations to limit geitonogamous self-pollination. The contrast between the spectral reflectance of the lateral petals and UV-absorbing patches on the labellae in these generalised food deceptive orchids is proposed to be a case of generalised pollen mimicry. This chapter also reports high levels of pollen transfer efficiency in species pollinated by *Xylocopa* bees and cetoniid beetles relative to those species pollinated by small solitary bees; as well as the results of controlled pollination experiments which show that three species are self-compatible, but dependent on pollinator visits for seed production. Traits associated with bee- and beetle-pollination are summarized and then used to develop predictions about pollination systems in other *Eulophia* species.

INTRODUCTION

Bee pollination in orchids

Bees are the most widespread and important pollinators of plants (Faegri & van der Pijl 1979). This also holds for the orchids (van der Pijl & Dodson 1966, Dressler 1981, chapter 1). Bees visit flowers for a variety of rewards, including nectar collected by both male and female bees as well as pollen collected by females to provision their nests (Proctor *et al.* 1996). In the case of orchids, pollen is almost always unavailable as a reward, being bound up as pollinia. As a result a number of species produce pseudopollen which is used as a substitute reward by pollinating bees (Dressler 1981, Davies *et al.* 2002, Davies & Turner 2004). In addition, bees may collect alternative rewards from orchids, such as oils for food or nest building and resins for nest building (Proctor *et al.* 1996), while male Euglossine bees collect scent compounds for use in their courtship displays (Cameron 2004). Bees are also often the victim of floral deception by orchids (Jersáková *et al.* 2006, chapter 1). To date, all *Eulophia* species examined are deceptive with no reward being offered to the pollinating insects.

The importance of different groups of pollinators in the Orchidaceae has not been well documented. However, using the catalogue of known relationships between orchids and their pollinators published by van der Cingel (1995, 2001), I was able to estimate the contributions of different animal groups to orchid pollination (see chapter 1). According to this analysis, bees are involved in the pollination of 41% of orchid species whose reproductive biology has been studied. Given that auto-pollinating species are probably over represented in this analysis owing to the relative ease of documenting these systems, I compared animal-mediated pollination systems just among xenogamous orchids [Table 1 of chapter 1]. Thus defined, bees pollinate 58% of documented species, followed in order of importance by wasps, flies, birds, settling moths, hawkmoths, butterflies and beetles. The latter have been found as pollinators of just 1.6% of studied species. Generalists (defined very broadly as orchids pollinated by two or more of the defined groups) account for just 3% of orchids, and in many of these instances bees are also important pollinators of the generalist species. Van der Pijl & Dodson (1966) estimated that 60% of orchid species are pollinated by bees, a remarkably similar estimate to my more recent one given that we have the benefit of nearly 40 years of additional data.

Orchid pollination by beetles

In contrast to bee pollination, beetle pollination has been considered less common in the Angiosperms, although Bernhardt's (2000) review of beetle pollination in angiosperms lists 180 species in 34 families which are specialized for beetle pollination and 98 species in 22 families where beetles contribute to generalist pollination systems. Faegri & van der Pijl (1979) suggested that the notion that beetle pollination is scarce may be a product of the focus, until recently, on European pollination systems where beetles are less important pollinators. There is increasing evidence that beetles are important pollinators in southern Africa (e.g. Steiner 1998, Steiner 1998, Goldblatt *et al.* 1998, Goldblatt *et al.* 2000, Johnson & Midgley 2001, Goldblatt *et al.* 2001, Johnson *et al.* 2004, Goldblatt *et al.* 2004, Goldblatt *et al.* 2005, Peter & Johnson 2006, Johnson *et al.* 2007).

Beetles seek out flowers for three forms of reward: edible rewards including nectar, pollen, stigmatic exudates and flower parts; as a rendezvous sites for mating purposes; or a temperature reward with the temperature of the flower being elevated above ambient (Bernhardt 2000). Beetle-pollinated flowers are rarely deceptive and in only a few orchid species (e.g. Steiner 1998, Peter & Johnson 2006a) and *Orchidantha inouei* (Lowiaceae; Sakai & Inoue 1999) have been demonstrated to be non-rewarding. Of the beetle-pollinated species included in van der Cingel's work (1995, 2001) and the current study, seven are deceptive and eight rewarding.

Flowers or inflorescences specialised for pollination by beetles are typically large, open and "unspecialised" (Faegri & van der Pijl 1979) forming one of four flower types: chamber blossoms, painted bowls, brush flowers and bilabiate flowers, the latter being limited to the few orchids pollinated by beetles and *O. inouei* (Bernhardt 2000). Flower scents among beetle-pollinated flowers include sweet and pleasant, strong fermenting fruit or decaying animal and dung scents. Flowers may also be unscented to the human nose (Johnson & Midgley 2001). Similarly, the colours of specialised beetle-pollinated flowers run the gamut from dull white and greens to dark browns and purples to bright yellows, blues and even reds (Faegri & van der Pijl 1979, Proctor *et al.* 1996).

Only a handful of orchid species have been shown to be pollinated by beetles. These make up just 1.6% of the species with known pollination systems listed by van der Cingel (1995, 2001). In addition to these 14 documented cases (which include the four cases of beetle

pollination in *Eulophia* identified in this thesis), beetles are implicated in a further 10 instances of generalist systems (Nilsson 1978, Nilsson 1981, Gutowski 1990, Pellegrino *et al.* 2005, Schatz 2006, Johnson *et al.* 2007). A detailed description of beetle pollination in *Eulophia foliosa* is given by Peter & Johnson (2006a [chapter 3]).

Pollination of Eulophia

As noted in chapter 1, little has been known about the pollination biology of the genus *Eulophia*. Prior to this research only one species pollinated by *Xylocopa* carpenter bees (Lock & Profita 1975) and a handful of auto-pollinating species from Zambia (Williamson 1984) have had their pollination biology examined in any detail.

Given the virtual absence of prior studies, I used pollination syndromes to develop initial hypotheses about pollinators for *Eulophia* species. Such hypotheses are essential when working on the pollination of deceptive orchids, because pollinators are very rarely caught directly on the orchid flowers. Syndrome hypotheses allow likely pollinators to be targeted visiting flowers of nearby rewarding species. Several *Eulophia* species have flowers which conform to the classical bee-pollination syndrome (van der Pijl 1961, Faegri & van der Pijl 1979). These species have deep zygomorphic flowers that are mechanically strong and have a landing platform. Flower colours are frequently "lively" and include colours such a yellow and blue often with nectar guides, while the scent of such flowers is "fresh" but not particularly strong. The sexual organs in these flowers are often hidden as is the nectar (van der Pijl 1961, Faegri & van der Pijl 1979). This syndrome proved to be accurate in forecasting which *Eulophia* species are pollinated by bees.

Given the wide diversity of beetle pollination systems, it was harder to characterize a single floral syndrome associated with beetle-pollination (see above). As a result, most instances of beetle-pollination in the genus were discovered either by searching very generally for insects bearing *Eulophia* pollinaria or by making direct observations of beetles on flowers of *Eulophia* species.

Pollinators for several *Eulophia* species are described in other chapters of this thesis. These include *E. zeyheriana* a Batesian mimic of *Wahlenbergia cuspidata* pollinated by *Lipotriches* (Halictidae) bees (Peter & Johnson 2008 [chapter 2]); *E. foliosa* pollinated by small click beetles (Peter & Johnson 2006a [chapter 3]); two forms of *E. parviflora*, one form pollinated

by *Amegilla fallax* (Anthophorinae), the other form pollinated by Cetoniinae beetles (Scarabaeidae, [chapter 6]). Pollinators for the remaining species are described in the present chapter. The value of these case histories, beside their contribution to our understanding of the natural history of the genus, is that they will eventually allow for comparative analyses of the evolution of pollination systems in a phylogenetic context.

Post removal pollinarium reconfiguration

Following removal by pollinators, pollinaria may undergo strikingly rapid changes such as bending, shrinking and dropping their anther caps after a specific interval. These changes in pollinarium configuration caught Darwin's attention who hypothesised that these changes are adaptations to limit pollinator mediated self-pollination (1867). A number of additional examples of pollinarium reconfiguration in the orchid and asclepiads have been described since Darwin's work, but these descriptive studies have not tested Darwin's hypothesis. Johnson *et al.* (2004b [appendix 1b]) provide evidence that supports Darwin's hypothesis, while Peter and Johnson (2006b [chapter 4]) tested Darwin's hypothesis using a number of *Eulophia* species as well as other orchids and asclepiads. They show that in 18 out of 19 species, pollinarium reconfiguration times exceed the visit times to the inflorescences by the respective pollinators which should effectively protect these species from facilitated self-pollination. Given the rapid movements and visit times of bees relative to beetles, I predicted that pollinarium reconfiguration should generally be quicker in bee- than in beetle-pollinated species.

Floral spectral reflectance

Flower colour has been considered one of the key traits of most pollination syndromes (van der Pijl 1961, Faegri & van der Pijl 1979) and is often the key mode of attraction for pollinators (Proctor *et al.* 1996). However, the associations between spectral reflectance and different insect visitors are also often not clear-cut, suggesting that colour may serve only as a very coarse filter of floral visitors (Chittka & Menzel 1992, Gumbert *et al.* 1999).

Insects and other pollinating animals have diverse responses to different wavelengths with sensitivities ranging from less than 300 nm to more than 700 nm (Briscoe & Chittka 2001) including colours, particularly ultraviolet, that are not perceptible to humans. Flowers frequently have contrasting colours including ultraviolet. Hypotheses to explain these contrasting colours include the nectar guide hypothesis, pollinator conspecific mimicry

hypothesis and the pollen and anther mimicry hypothesis (Heuschen *et al.* 2005). This latter explanation holds that some plants have signals typically in the centre of the flower that advertise the presence of pollen reward (Lunau 2000, Heuschen *et al.* 2005). In contrast to this generalised pollen mimicry, there is also evidence for more specific pollen mimicry, where pollen mimicking patches on the labellae of deceptive orchids is a key component of the mimicry of the rewarding model species (Nilsson 1983, Peter & Johnson 2008).

Pollen transfer efficiency

As explained by Peter & Johnson (submitted [chapter 5]), PTE can be considered a population level measure of the proportion of pollen removed from anthers that is subsequently deposited on conspecific stigmas (Johnson *et al.* 2005, Harder & Johnson 2008). In orchids it is possible to rapidly assess rates of pollinaria removal or pollinia deposition on stigmas by pollinators because of the relatively large size of pollinia and massulae relative to typical granular pollen.

Nectar rewards are expected to increase overall visitation rates to plants in a population (Johnson *et al.* 2003b [appendix 1a]) and as a result, PTE in rewarding species should be higher than in their deceptive relatives. Peter & Johnson (submitted [chapter 5]) provide data to support this idea. Other aspects of pollination biology, such as pollinator type, and whether or not mimicry is involved, would similarly be expected to influence PTE.
For example in floral Batesian mimicry, the deceptive plant is subsidised by the positive conditioning the reward model provides to the mimic and the PTE of these species is expected to be higher than their generalised food deceptive relatives. In general, I expected bee-pollinated species to have higher levels of PTE than beetle-pollinated species, on the basis that morphological interactions between bees and flowers would be more precise than those involving beetles, and thus involve less pollen wastage.

Aims

The aim of this chapter was to extend our knowledge of pollination in the genus *Eulophia* by documenting pollination case histories for several species pollinated by either bees or beetles, and to analyze patterns of post-removal pollinarium reconfiguration, floral spectral reflectance, pollen transfer efficiency and the breeding systems of these species.

MATERIAL AND METHODS

Study sites

Populations of all *Eulophia* and *Acrolophia* species encountered in southern and eastern South Africa were examined (Table 1, Map 1 and 2 at the end of the thesis). Times spent observing populations of the different species are given in Table 1.

Table 1: Estimated time spent observing populations of each of the study species. Species in bold have had their pollinators identified.

Current chapter	
E. angolensis	22 hrs
E. cucullata	36 hrs
***E. ensata* Yellow form**	20 hrs
***E. ensata* Cream Form**	10 hrs
E. ovalis	18 hrs
E. speciosa	34 hrs
E. streptopetala	15 hrs
E. welwitschii	19 hrs
Other chapters in the thesis	
***A. cochlearis*[1]**	38 hrs
***E. foliosa*[2]**	50 hrs
***E. parviflora* Long-spurred form[3]**	109 hrs
***E. parviflora* Short-spurred form[3]**	63 hrs
***E. zeyheriana*[4]**	79 hrs
1.	3.
Other taxa examined	
A. micrantha[1]	10 hrs
A. capensis[1]	10 hrs
E. aculeate	3 hrs
E. calanthoides	1 hr
E. clitellifera	4 hrs
E. hereroensis	1.5 hrs
E. leontoglossa	10 hrs
E. macowanii	1 hr
E. odontoglossa	3 hrs
E. parvilabris	1.5 hrs
E. petersii	6 hrs
E. tuberculata	3 hrs

[1] chapter 5, [2] chapter 3, [3] chapter 6, [4] chapter 2

The study species

Eulophia speciosa (R. Br. Ex Lindl.) Bolus (Fig. 1A, B) has large showy yellow flowers arranged on relatively tall inflorescences reaching 1.5 m. The floral display is due primarily to the two bright yellow lateral petals and stout labellum. The labellum is prominently marked with dark purple nectar guides radiating from a short slit like spur. The sepals are green and for the most part hidden behind the lateral petals. This species is normally scentless to humans although some plants I have examined have a sweet but subtle scent. The leaves of this species are succulent. *E. speciosa* is a common species found in South Africa mostly along a narrow coastal strip never more than about 1 km from the sea growing in disturbed ground and stabilised beach sand. In the north of KwaZulu-Natal and in tropical Africa, the range of this species extends inland.

Eulophia streptopetala Lindl. (Fig. 2A, B) is another relatively large and showy species with inflorescences reaching 1.5 m. A few flowers are found open at a time. The petals give this species its specific epithet, being twisted forward. Abaxially the petals are bright yellow and form a major part of the display of this species. Adaxially the petals are much paler to almost white in some plants. The labellum is a similar bright yellow adaxially with brown to red side lobes. There is a short sac-like spur at the base of the column. Sepals are green, densely mottled with dark brown. No flower scent was obvious to the human nose. The leaves are broad, thin tissued and plicate. This is a common species growing in thicker vegetation such as the scrubby margins of forests. It extends from near Port Elizabeth in the south through the eastern part of South Africa into tropical Africa.

Eulophia cucullata (Sw.) Steud. is a spectacular *Eulophia* although inflorescences only attain about 50 cm and bear only a few large flowers (Fig. 3). The lateral petals and labellum are bright pink, while the sepals are darker purple. The labellum forms a large sac-like pouch below the column. The inner wall of this pouch is yellow flecked with very dark purple. There are two rhomboid-shaped ridges projecting from the labellum. The flowers of this species have a strong "chemical" or "plastic" scent. In South Africa this species is found in moist coastal grassland from near Durban, northwards into tropical Africa where it is a common species.

Eulophia angolensis (Rchb. f.) Summerh. produces tall, many-flowered inflorescences that attain over 2 m in height. Flowers are densely packed on the inflorescences with all parts bright yellow (Fig. 4A). The flowers are strongly and sweetly scented, so much so that large populations produce a heady scent in the swamps in which this species grows. Flowers stained with neutral red indicate that the ridges on the labellum are the site of scent production in this species (Fig. 4). *E. angolensis* is found from the north-eastern parts of the former Transkei through the coastal parts of KwaZulu-Natal and thence inland to the lowveld of Mpumalanga and into tropical Africa.

Eulophia ovalis Lindl. is a smaller species with sparse inflorescences reaching about 50 cm. Relatively few flowers (often only one) are open on an inflorescence at any one time. Sepals are typically brown to green while the lateral petals and labellum are bright white to pale cream. Two subspecies are currently recognised. This research focuses on subspecies *ovalis* found from the coast to the foot of the Drakensberg mountains and throughout the eastern parts of South Africa. Subspecies *bainesii* (Rolfe) A.V. Hall is rarer, having larger flowers and a shorter spur. There is no obvious floral scent.

Eulophia ensata Lindl. is a common species found at low and mid altitudes throughout eastern South Africa. The flowers of this species are crowded into dense capitate inflorescences (Figs. 6 A and B). Two different colour forms have been observed over the course of this study. The commonly encountered bright yellow form is found throughout KwaZulu-Natal and the Eastern Cape. As part of this research, populations of plants with exclusively cream coloured flowers (Fig. 7) were observed in the far north eastern parts of KwaZulu-Natal. There are no obvious morphological differences between the two colour forms. Neither form is scented to the human nose.

Eulophia welwitschii (Rchb. f.) Rolfe is similar in many respects to *E. ensata*, with dense crowded inflorescences (Fig. 8A) although less dense than in the case of *E. ensata*, while the flowers of *E. welwitschii* are larger than those of *E. ensata.* This species occurs in huge populations (Fig. 8B) at mid and higher altitudes through the eastern parts of South Africa. Unlike *E. ensata*, the cream flowers have strongly contrasting tepals and labellum, the latter being very dark maroon, almost black. In some populations near the southern Drakensberg there are individuals without the dark labellum, it being the same colour as the tepals (Fig. 8C). There is no obvious scent.

Observations were also made on a number of other species including *Eulophia aculeata* (L.f.) Spreng. subsp. *aculeata*, E. a. subsp. *huttonii* (Rolfe) A.V. Hall, *E. calanthoides* Schltr., *E. clitellifera* (Rchb. f.) Bolus, *E. hereroensis* Schltr., *E. horsfallii* (Batem.) Summerh., *E. leontoglossa* Rchb. f., *E. macowanii* Rolfe, *E. odontoglossa* Rchb. f., *E. parvilabris* Lindl., *E. petersii* (Rchb. f.) Rchb. f. and *E. tuberculata* Bolus.

Pollinators

Pollinators were collected in the vicinity of flowering plants at each of the study sites. As most species of *Eulophia* are deceptive, visits by bee pollinators are rarely observed. Most pollinators were therefore collected while they were visiting other rewarding plants near to the orchids. In contrast, the majority of the beetle pollinators were collected on the orchid inflorescences directly.

Insects bearing pollinia were killed in ethyl acetate killing jars with care taken to avoid dislodging the pollinia (the viscidium glue is rapidly dissolved by ethyl acetate fumes), mounted and identified. Insects are lodged in the collection of the first author and the Albany Museum, Grahamstown.

Pollinarium reconfiguration

Pollinarium reconfiguration times were recorded for most species. In species having a rapid bending mechanism, the end point of the reconfiguration is obvious and easily timed. In species with a slow bending action, rates of change of the angles of the pollinaria were determined with a protractor and plotted against time to determine the end points (see Johnson & Nilsson 1999, Peter & Johnson 2006b [chapter 4] for details). Anther cap retention occurs in some species, the details of this reconfiguration in *E. foliosa* is given by Peter & Johnson (2006a [chapter 3]). Where anther cap retention was observed, the duration from pollinarium removal from the flower to the dropping of the anther cap was recorded. During this time, the pollinarium and anther cap were constantly agitated in a light air current so that the end point (dropping of the anther cap) could be determined.

Visitation rates and Pollen Transfer Efficiency (PTE)

In large populations, one flower is typically sampled randomly from each plant for the determination of PTE. However for most of the species examined here, the number of

individual plants in a population was low and in these cases, it was possible to score the pollinaria removal and pollinia deposition for every flower on each inflorescence encountered in the population. The average number of pollinia deposited on stigmas was divided by the number of pollinia removed (pollinaria multiplied by two – each pollinarium comprising two pollinia) and expressed as a percentage to determine PTE (Johnson *et al.* 2005):

$$\text{PTE} = \frac{\text{average number of pollinia deposited}}{\text{(average number of pollinaria removed x 2)}} \times 100$$

In a number of instances flowers with missing anther caps were observed, indicating a failed visit to the flower. These were scored and expressed as a percentage of the flowers showing visitation (deposition, removal or disturbed anther caps) to estimate a rate of pollination failure.

Flower colour

Flower colours were analysed quantitatively with an Ocean Optics spectrophotometer. Measured colours were summarised according to the model of Chittka (1992). Flower pattern was determined qualitatively using UV photography. Details of both the spectrophotometery and UV photography are given by Peter & Johnson (2008 [chapter 2]).

There is evidence that some beetles are sensitive to the red parts of the spectrum (Dafni *et al.* 1990, Johnson *et al.* 2004a) and that some beetles posses tetrachromatic vision (Briscoe & Chittka 2001), in which case the Chittka (1992) model might not be ideal. However I elected to use this model to analyze the colours of floral parts of *Eulophia* flowers for a number of reasons: this model is currently the most widely applied and understood model which makes the data presented here directly comparable to other published studies; trichromatic vision of hymenoptera which forms the basis of the model seems phylogenetcally conserved and widespread in the insects (Briscoe & Chittka 2001) and probably applies to many flower visiting beetles, the observations above notwithstanding; the majority of the inferences made about the contrasting flower colours apply to bees; and red is rare in *Eulophia* and beetle-pollinated *Eulophia* species in particular, with only the short spurred form of *E. parviflora* having a reddish brown component to the interior of their flower where this colour is unlikely to contribute to the primary attraction.

Breeding systems

To establish the dependence of plants on pollinators, breeding system studies were conducted for *E. speciosa*, *E. angolensis* and *E. welwitschii*. Breeding systems for the bee-pollinated *E. zeyheriana*, the beetle-pollinated *E. foliosa*, the rewarding *Acrolophia cochlearis* and the long- and short-spurred form of *E. parviflora* are reported elsewhere (chapter 2, chapter 3, chapter 5 and chapter 6 respectively). In all cases, inflorescences where bagged to exclude pollinators and the flowers either self-pollinated, cross-pollinated with pollen from other plants in the population, or left untreated to test for auto-pollination.

RESULTS

Insect pollinators and pollinarium reconfiguration

Eulophia speciosa was found to be pollinated exclusively by large *Xylocopa flavorufa* carpenter bees (Fig. 1C), the pollinaria being attached to the bee between its antennae (Fig. 1D). Unfortunately the majority of the insects bearing pollinaria were collected in the vicinity of cultivated plants grown outside the natural range of the plants. The majority of these were captured in the Botanical Gardens of the University of KwaZulu-Natal in Pietermaritzburg, approximately 50 kilometres from the nearest known naturally occurring plants. In four instances, bees were observed probing the flowers and removing the pollinaria. Other bees bearing pollinaria were collected while foraging on a species of *Clerodendron* growing nearby. Smaller species of *Xylocopa*, including *X. caffra* and *X. flavicollis*, were common at this site, but none of the many insects collected and inspected bore pollinaria or viscidia.

Other observations of visits to flowers of *E. speciosa* were made on a plant cultivated in the Department of Botany, Rhodes University, Grahamstown approximately 40 kilometres outside of the natural range of this species. A visit was also observed to flowers of a plant growing in a garden in Kloof, right on the edge of the natural distribution of this species. In natural populations including those of the Bayhead Natural Heritages site in Durban and Kenton-on-Sea in the Eastern Cape, bees were observed to inspect the flower closely but they did not alight on nor probe the flowers. In the very large population at the Bayhead Natural Heritage site, a number of different individuals were observed to hover and closely inspect a long succession of inflorescences before losing interest and flying away.

Similar observations were made on cultivated plants growing in Grahamstown (S. Ripley pers. comm.) where two approaches to the flowers were seen.

In addition to the four bees collected bearing pollinaria in the Botanical Gardens at the University of KwaZulu-Natal, Pietermarizburg and the bee collected in Grahamstown, a bee carrying pollinaria was collected in the vicinity of wild plants at Kenton-on-Sea while feeding on an exotic species of *Senna* (Fabaceae).

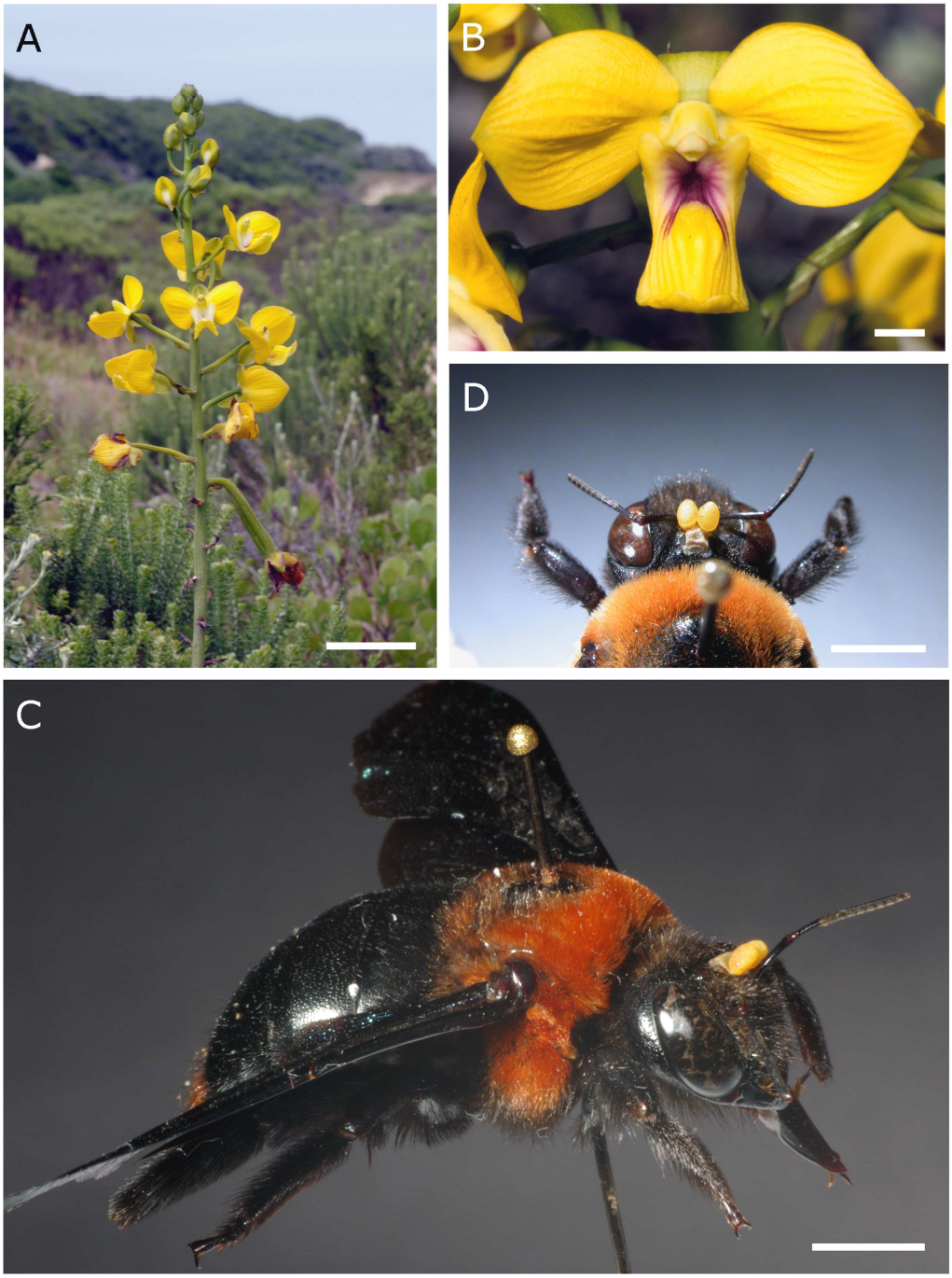


Figure 1: A) *Eulophia speciosa* produces large showy inflorescences made up of a number of large flowers (B). This species is pollinated by C) *Xylocopa flavorufa* with the pollinaria being attached between the antennae of these bees (D). Bar: A = 5 cm; B, C and D = 5mm.

Table 2: Recorded pollinators of *Eulophia* and *Acrolophia* species[1].

Pollinator	Family	Sex	Sites[2]	Number of insects observed or collected	Number of insects with pollinaria or viscidia	Number of insects approaching or visiting
E. zeyheriana [chapter 2]						
Lipotriches sp	Halictidae	Males	20, 28	70 (46 collected)	34 of those collected	2
E. foliosa [chapter 3]						
Cardiophorus obliquemaculatus	Elateridae	unknown	2, 14, 18, 27, 34, 39	102	26	19
Atricelaphinis tigrina	Cetoniinae, Scarabaeidae	Both sexes	30	7	4	4
	Various Coleoptera	unknown	18, 34, 39	48	0	48
	Pompilidae	unknown	2, 18	2	0	2
E. speciosa						
Xylocopa flavorufa	Xylocopinae, Apidae	9 F	2, 6, 12, 13, 41, 42	37	9	20
Xylocopa flavicollis	Xylocopinae, Apidae	5 F, 1 M	12, 13, 24, 41	6	0	0
Xylocopa caffra	Xylocopinae, Apidae	3 F	6, 12, 41	3	0	0
E. streptopetala						
Megachile cincta	Megachilidae	4 M, 2 F	23, 26, 37, Note 3	7	4	0
Megachile felina	Megachilidae	2 F	26, 37	4	1	0
Megachile sp. (CIP 3878)	Megachilidae	Females	37	1	1	0
E. cucullata						
Xylocopa flavicollis	Xylocopinae, Apidae	Females	22, 23, 24	21	6	0
Xylocopa hottentotta	Xylocopinae, Apidae	3 M, 7 F	22, 23, 24	20	3	0
E. angolensis						
Campsomesiella calebs	Scoliidae	Female	17	1	1	1
Xylocopa flavicollis	Xylocopinae, Apidae	Females	17	14	3	10

E. ovalis subsp ovalis						
Lassioglossum sp.	Halictidae	2 F, 3 M	32	5	1	0
***E. ensata* (Yellow)**						
Cytothyrea marginalis	Cetoniinae, Scaraebidae	Both sexes	39	18	4	0
Atrichelaphinis tigrina	Cetoniinae, Scaraebidae	Both sexes	39	20	2	7
Leucocelis cf. amethystina	Cetoniinae, Scaraebidae	Both sexes	39, 36	44	9	2
Allodape rufogastia or *A. exoloma*	Bee	Female	18	1	1	1
***E. ensta* (Cream)**						
Leucocelis cf. amethystina	Cetoniinae, Scaraebidae	Both sexes	23	19	4	2
E. welwitschii						
Atrichelaphinis tigrina	Cetoniinae, Scaraebidae	Both sexes	31, 33	54	8	25
Leucocelis cf. amethystina	Cetoniinae, Scaraebidae	Both sexes	10	6	4	2
***E. parviflora* (Long-spurred form)** [chapter 6]						
Amegilla fallax	Anthophorinae, Apidae	Both sexes	40, 41	89	20	7 (9)
***E. parviflora* (Short-spurred form)** [chapter 6]						
C. marginalis	Cetoniinae, Scarabaeidae	Both sexes	2, 15, 33, 35, 36, 41	589	61 (9)	9 (2)
E. cristata (Lock & Profita 1975)						
Xylocopa olivacea	Xylocopinae, Apidae	Not recorded	Southern Ghana	"A number"	"A number"	"A number"
Acrolophia cochlearis [chapter 5]						
Colletes claripes	Colletidae	8 M (+ 9)[4]	2	8 (+9)[4]	8 (+9)[4]	8 (+9)[4]
Colletes claripes	Colletidae	2 F	2	2	0	2

[1] This table is a summary of Appendix 1 of this chapter [chapter 7] and includes primarily the pollinarium/viscidium bearing insects or insects visiting flowers of the study species.
[2] Numbers correspond with those used in Map 1 & 2 at the end of this thesis.
[3] One specimen housed in AMGS bearing pollinaria collected at MacIlwaine, Salisbury (= Harare).
[4] An additional 9 male bees were observed bearing large pollinaria masses, patrolling and visiting the inflorescences, but were not collected.

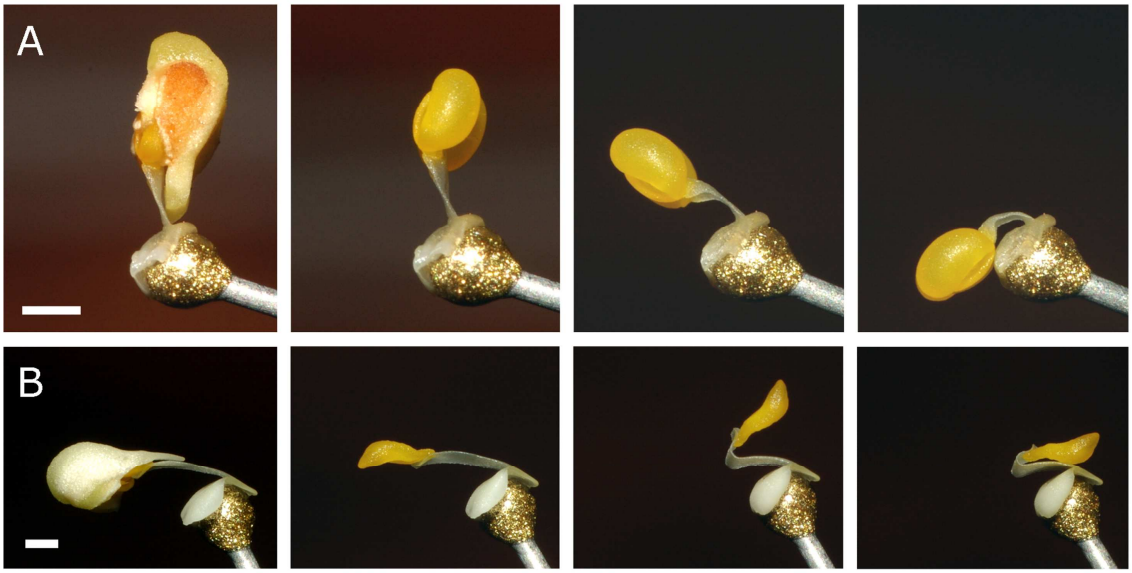


Figure 9: The two bending-type reconfiguration mechanisms identified so far in *Eulophia*. A) The less common *E. speciosa*-type reconfiguration bends from an upright position to a depressed position. B) The more common *E. streptopetala*-type of reconfiguration entails bending of the pollinarium from an initially depressed position through nearly 180° so that the pollinia are finally orientated in the opposite direction. Bar = 1 mm.

The reconfiguration mechanism of *E. speciosa* is distinctly different from the more common reconfiguration mechanism found in most other species of *Eulophia* and described below (termed *E. streptopetala*-type reconfiguration as opposed to this reconfiguration, designated *E. speciosa*-type reconfiguration). In *E. speciosa* the freshly removed pollinarium is orientated with the pollinia perpendicular to the viscidium (Fig. 9A). The stipe then goes through a period of reconfiguration, bending forward so that the pollinia are orientated forward in a position comparable to pollinia in freshly removed pollinaria of the species listed below as having the "typical" *Eulophia streptopetala*-type reconfiguration mechanism (Fig. 9B). This reconfiguration takes 115.0 seconds (SE = 7.2, n = 35). The pollinaria of *Eulophia clitellifera* and *Eulophia tuberculata* also undergo this form of reconfiguration with average reconfiguration times of 32.3 s (SE = 1.5, n = 25) and 209.9 s (SE = 16.0, n = 7) respectively.

Eulophia streptopetala is pollinated by very large megachilid bees including *Megachile cincta* (Fig. 2C), an unidentified species of *Megachile* (Fig. 2D) and *Megachile felina* (Fig. 2E). Initially a very large bee (*M. cincta*) bearing unusual pollinaria was collected on a yellow *Crotalaria* species (Fabaceae) near Manzengwenya in Maputaland. It was not until four more *Megachile* bees were collected at Drummond in the KwaZulu-Natal midlands that the unusual elongated pollinia were identified as belonging to *E. streptopetala*. An

additional specimen of *M. cincta* bearing a distinctive *E. streptopetala* pollinarium and collected in Harare, Zimbabwe was found in the collection of the Albany Museum, Grahamstown.

At the Drummond site a male *M. cincta* bee was collected bearing four pollinaria which had already had their pollinia deposited or groomed off as well as a complete pollinarium with pollinia orientated for deposition (Fig. 2C). While looking for the pollinator of *E. ovalis* at Cobham in the Drakensberg, another Megachilid species, *M. felina* was collected also foraging on a legume (a species of *Otholobium*), bearing the unmistakable pollinaria of *E. streptopetala*. This is intriguing as this site is approximately 100 km from the nearest known collection locality of *E. streptopetala*. Extensive searches of the nearby bush and forest margins failed to reveal any *E. streptopetala* plants. Other collections at this site included a number of individuals of *M. cincta*, none of which bore pollinaria.

The pollinaria of *E. streptopetala* are attached to the posterior edge of the bee's head. Initially the pollinia are orientated forwards on the long slender stipe (Fig. 9B). The stipe bends mid length, flipping the pollinia through nearly 180° such that they are then orientated to point backwards and can thus be hooked into the stigmatic cavity (see also Fig. 1 of chapter 4). As noted above, this has been termed the *E. streptopetala*-type of reconfiguration. On average, the reconfiguration of the pollinarium takes 106.0 seconds (SE = 19.3, $n_{flowers}$ = 30, $n_{individuals}$ = 19). Visits by the pollinators to the flowers were not observed and so it is not possible to compare visit times to the reconfiguration times of the pollinaria.

Eulophia cucullata pollinators were collected near the southern-most limit of this species distribution at Amatikulu Nature Reserve near the mouth of the Utukela (Tugela) River. This species is pollinated by medium sized *Xylocopa* bees including female *Xylocopa flavicollis* and male and female *Xylocopa hottentotta*. The pollinaria are attached dorsally to the posterior margin of the metathoracic segment of the pollinating carpenter bees. The stipe of the pollinarium in this species is short and broad, but undergoes a similar reconfiguration to that described for *E. streptopetala*. This reconfiguration takes 155 seconds (n = 2). No visits to the flowers were observed despite the high visitation rates (Table 3).

Figure 2: A) *Eulophia streptopetala* produces tall inflorescences with a succession of flowers (B) which have prominent yellow lateral petals and a yellow labellum with maroon side lobes. This species is pollinated by various species of Megachilidae including C) *Megachile cincta*, D) an unidentified species of *Megachile* and E) *Megachile felina*. Bar = 5 mm.

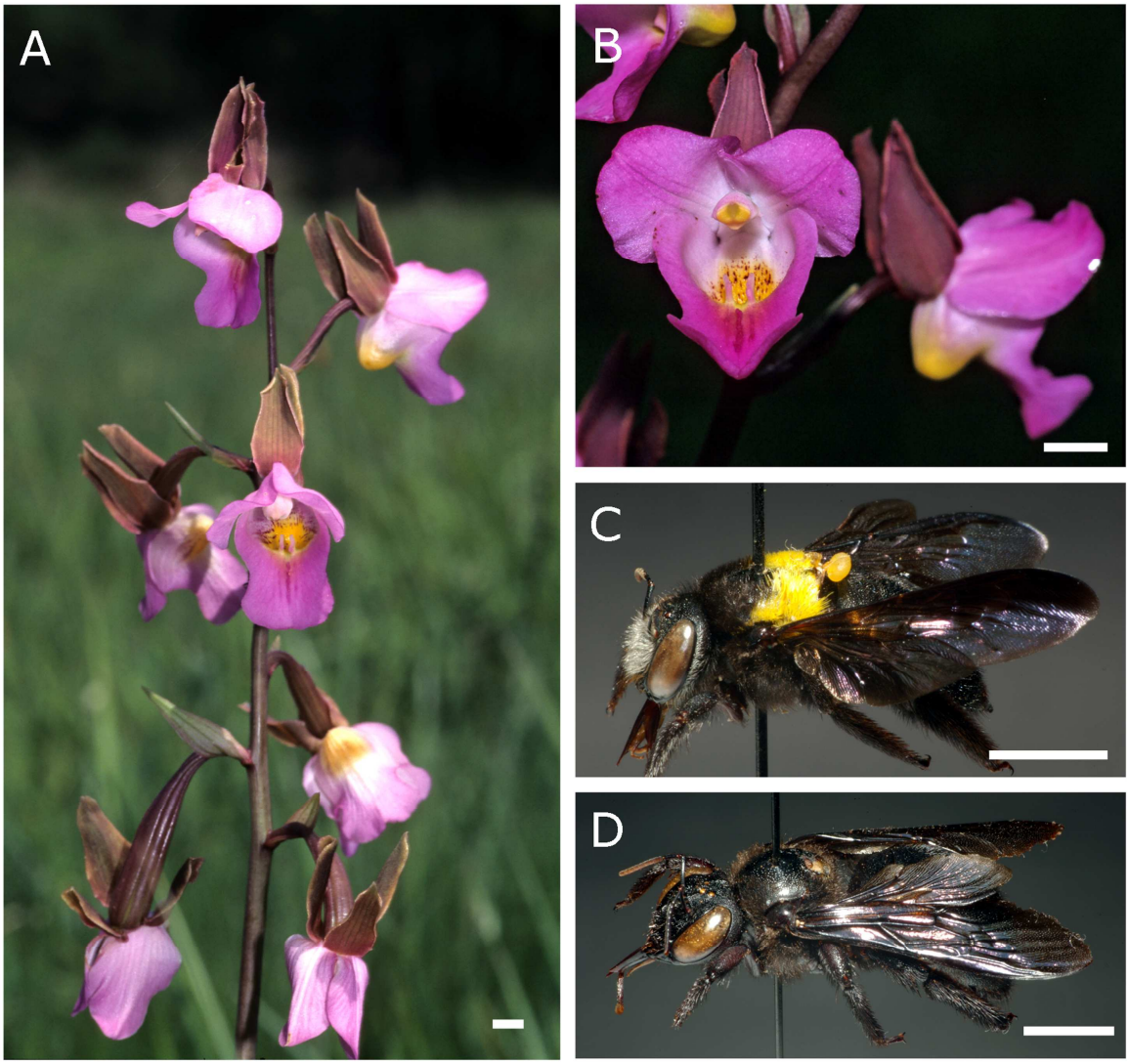


Figure 3: A) The inflorescences of *Eulophia cucullata* are relatively few flowered, the flowers (B) being bright pink with a prominent yellow base to the sac-like spur. This species is pollinated by C) *Xylocopa flavicollis* and D) *X. hottentotta*, this latter specimen bearing only a viscidium. Bar = 5 mm.

Eulophia angolensis is pollinated by similar medium-sized *Xylocopa* bees to those found pollinating *E. cucullata*, including *Xylocopa flavicollis*. A small number of these bees bearing pollinia were collected while they visited flowers of *E. angolensis* at Mpenjati on the KwaZulu-Natal south coast. In addition a very large Scoliid wasp (*Campsomesiella calebs*) was collected following a visit to a flower. This insect had removed the pollinarium from the flower that it visited. As is the case with *Eulophia cucullata*, the pollinaria of *E. angolensis* are attached to the dorsal edge of the metathoracic segment between the wings of the bee. The pollinaria undergo a similar reconfiguration to that described for *E. streptopetala*. This reconfiguration takes 72 seconds on average (SD = 35, $n_{flowers}$ = 53, $n_{individuals}$ = 15). The duration of the observed visits to inflorescences were not recorded, but the few visits seen lasted less than one minute.

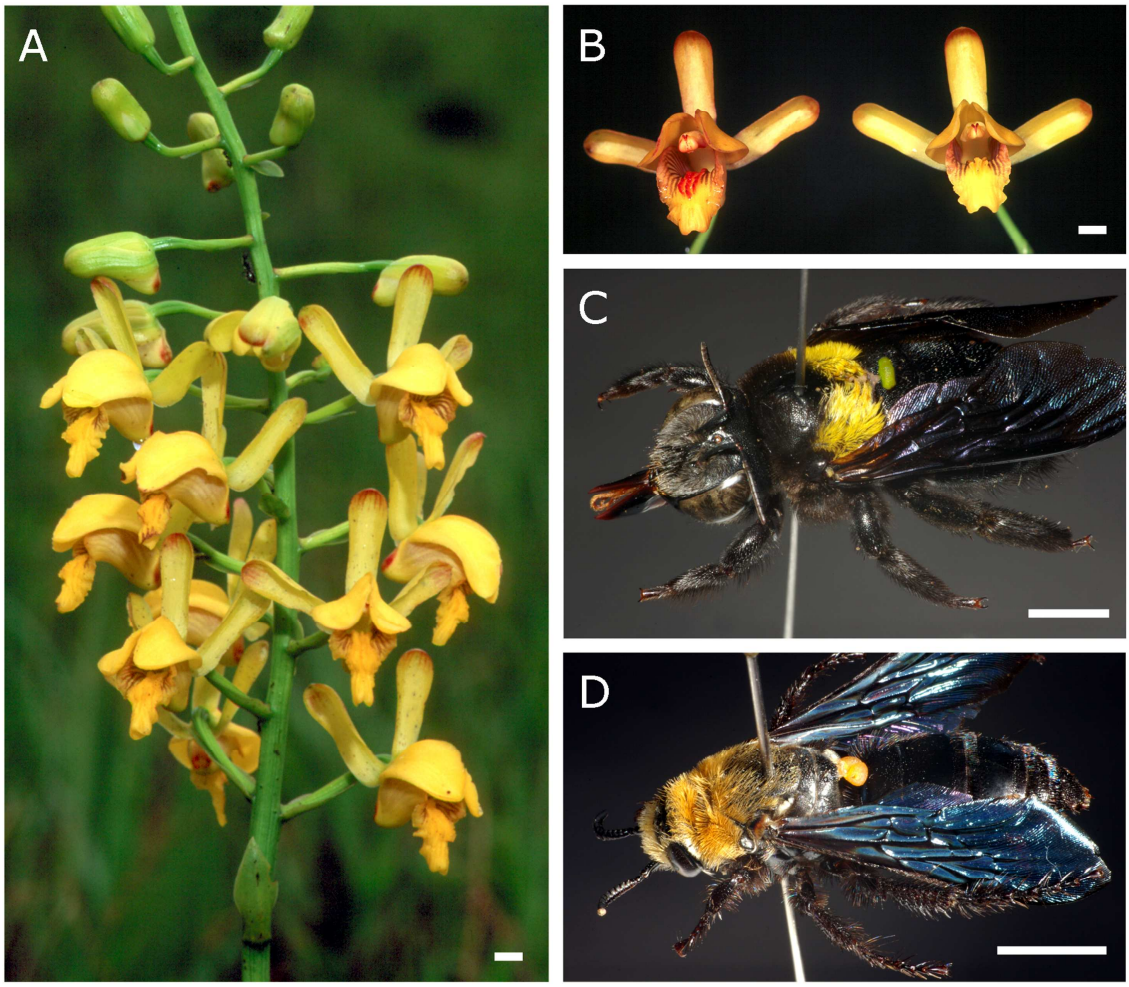


Figure 4: A) *Eulophia angolensis* produces large showy inflorescences with many open flowers. The flowers are sweetly scented, B) staining with neutral red indicates that the three lamellae ridges of the labellum are the site of scent production. *E. angolensis* is pollinated by C) *Xylocopa flavicollis*, with D) a scoliid wasp, *Campsomesiella calebs*, also being collected visiting the flowers. Bar = 5 mm.

A number of attempted visits by the large carpenter bee *Xylocopa flavorufa* (see Fig. 1) were also observed. In these instances the bees approached and grappled the end of the flower and attempted to enter the flower. However the two lateral petals forming the tube into which the bees crawl are too stiff to allow the entrance of such a large bee, effectively filtering out these bees as pollinators. The smaller pollinating *Xylocopa* bees and Scoliid wasps bearing pollinaria can, however, easily enter the flowers.

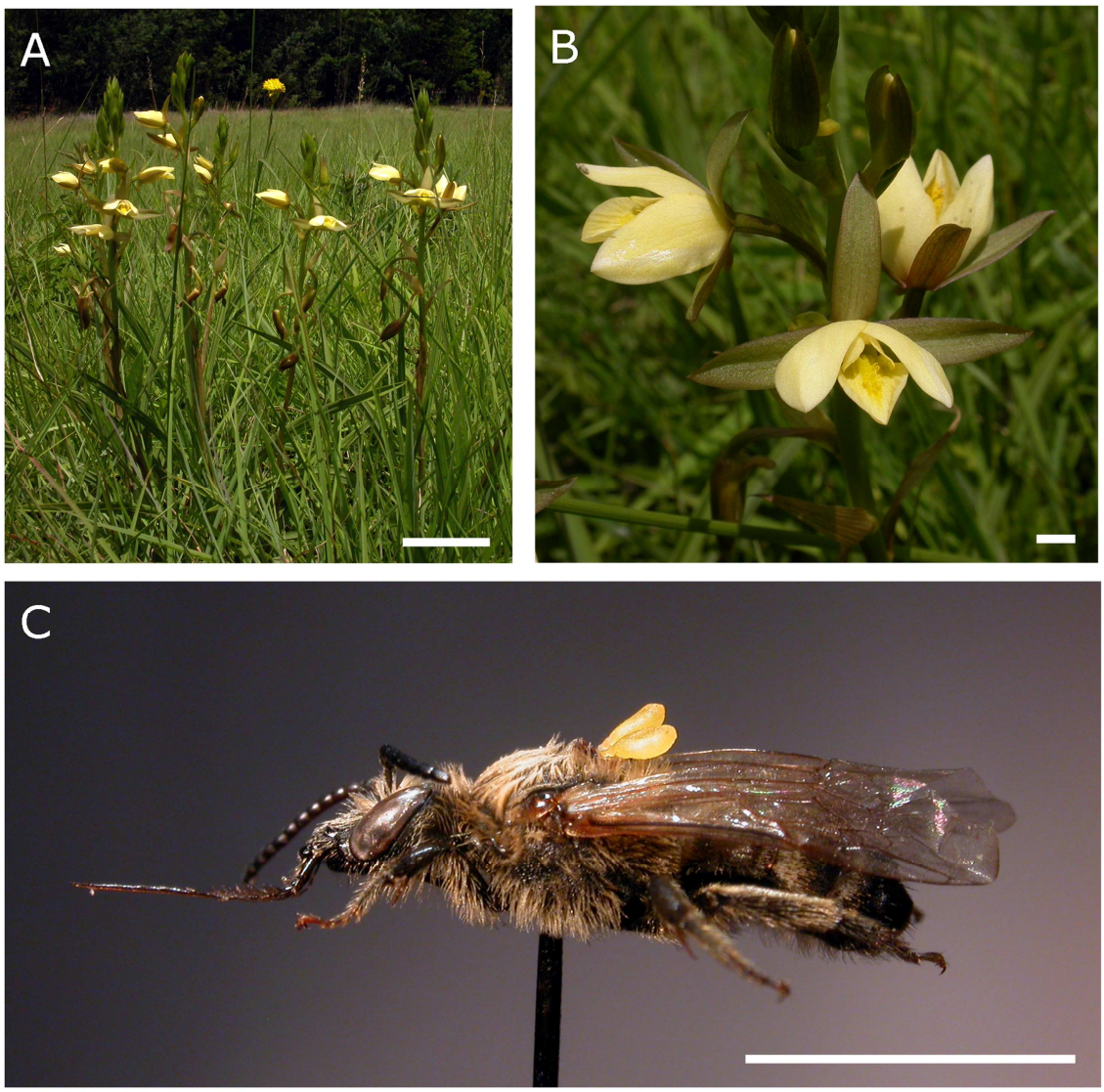


Figure 5: The infloresences of *Eulophia ovalis* subspecies *ovalis* are relatively few flowered often with less than three open flowers per inflorescence at any one time (A and B). Only one individual of a species of *Lasioglossum* (Halictidae) has been collected bearing the polliniaria of this subspecies. Bar: A = 5 cm; B and C = 5 mm.

Eulophia ovalis ovalis. Despite considerable observation effort at two sites examining *Eulophia ovalis*, only one individual of a *Lasioglossum* species (Halictidae) bearing the polliniaria of *Eulophia ovalis* sub-species *ovalis* (Fig. 5C) was collected at Wahroonga in the KwaZulu-Natal midlands. This bee was sheltering in *Gladiolus ecklonii* flowers during inclement weather. Four other bees of the same species were collected at the same time, but without evidence of bearing pollinaria or viscidia. Pollinarium reconfiguration in this species is rapid, occurring in 29.4 seconds on average (SD = 11.9, n = 9). A large number of bees and wasps were collected and inspected for pollinaria in the very large population of this species at Cobham in the southern Drakensberg. None of these Hymenoptera carried pollinaria.

***Eulophia ensata*.** Both the common yellow form (Fig. 6) and the undocumented cream form (Fig. 7) of *E. ensata* are pollinated by small cetoniid flower chafers. A total of 15 beetles were collected at Kloof as well as Thornville bearing pollinaria or viscidia of the bright yellow form, including primarily *Leucocelis* cf. *amethystina*[2] and *Cyrtothyrea marginalis* (Table 2). These were captured on inflorescences of the orchids and on nearby Asteraceae, primarily species of *Bekhaya* (Fig. 6C) and *Helichrysum nudiflorum*. A number of *Atrichelaphinis tigrina* beetles were also collected visiting the inflorescences, but none of these beetles bore pollinaria, nor did the many beetles of this species observed in the vicinity of the *E. ensata* plants.

In addition to the numerous beetles caught bearing pollinaria of yellow form, a small unidentified halictid bee (Halictidae) was found in the inflorescences of this species and a similar unidentified bee was observed on a plant found near Grahamstown (Fig. 6A, Arrow). A slightly larger anthophorid bee *Allodape* cf. *rufogastia* was collected in a flower of this species at Vernon Crookes Nature Reserve. This bee was seen to remove a pollinarium.

Anther cap retention occurs in this species. The anther caps take 154 seconds on average to drop (n = 18, range 75 to 285 seconds). Bending occurs during this time although this movement is complete by the time the anther cap is dropped.

Less time was spent making observations of the cream form. I collected four *Leucocelis* cf. *amethystina* beetles bearing pollinaria or viscidia of this form (Fig. 7C). These beetles were caught on the inflorescences of *Helichrysum nudiflorum*, the only large Asteraceae observed in the area.

[2] There are two similar species of *Leucocelis* including *L. amethystina* and *L. haemorrhoidalis*. These two species show a continuous range of morphological variation, but the absence of dorsal maculae (white spots) is consistent with *L. amethystine*, the name that will be used here. Definitive identification requires dissection of male genetalia (Holm & Marais 1992). From the plants' perspective, it seems unlikely that these two species have substantially different behaviours.

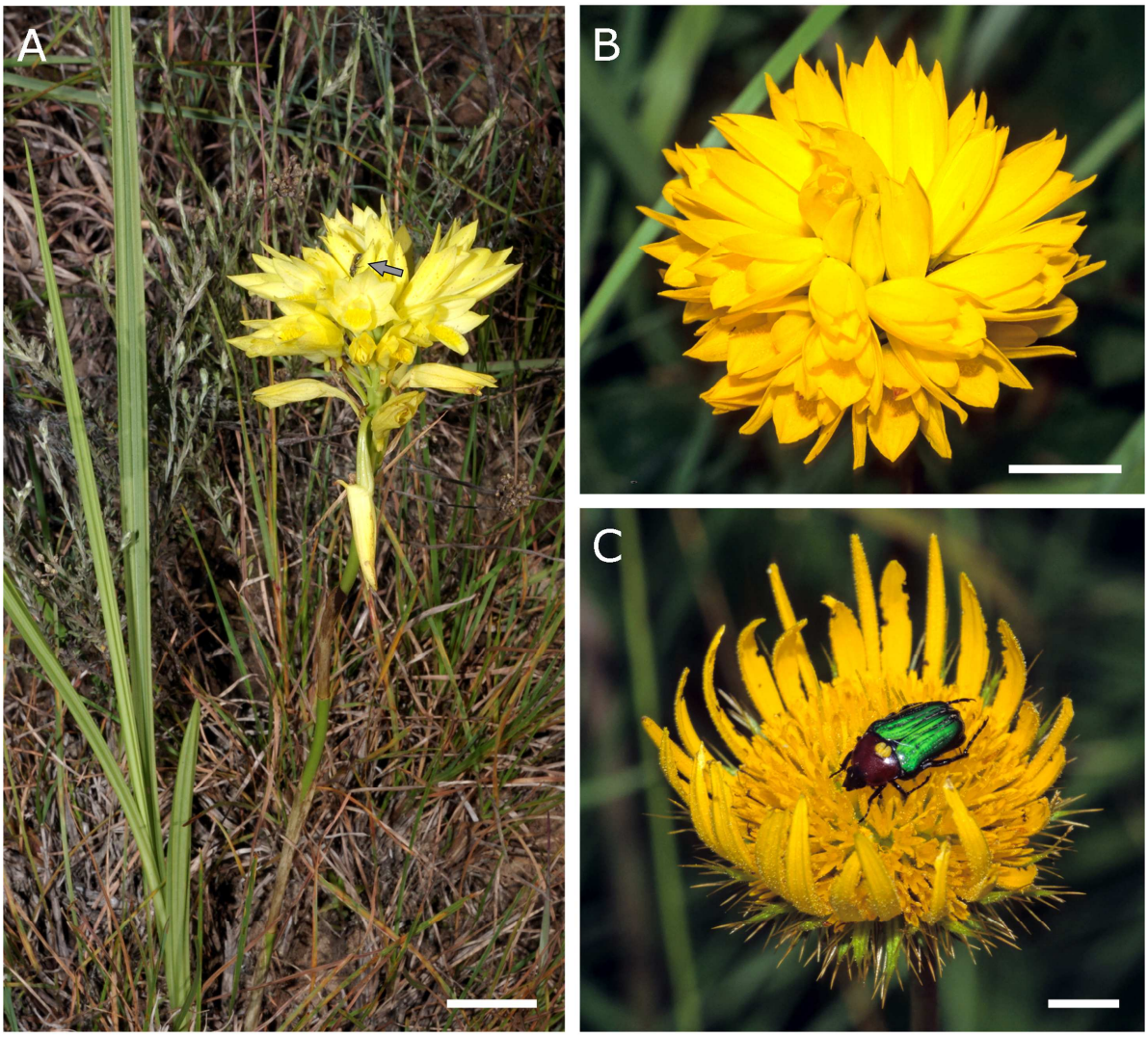


Figure 6: The yellow flowered form of *E. ensata* has dense head-like inflorescences (A and B) and is pollinated by Cetoniinae beetles including C) *Leucocelis cf. amethystina*, here visiting a *Berkhaya* species inflorescence. Bees have occasionally been found visiting the inflorescences of *E. ensata* (A; arrow). Bars: A & B = 2 cm, C = 5 mm.

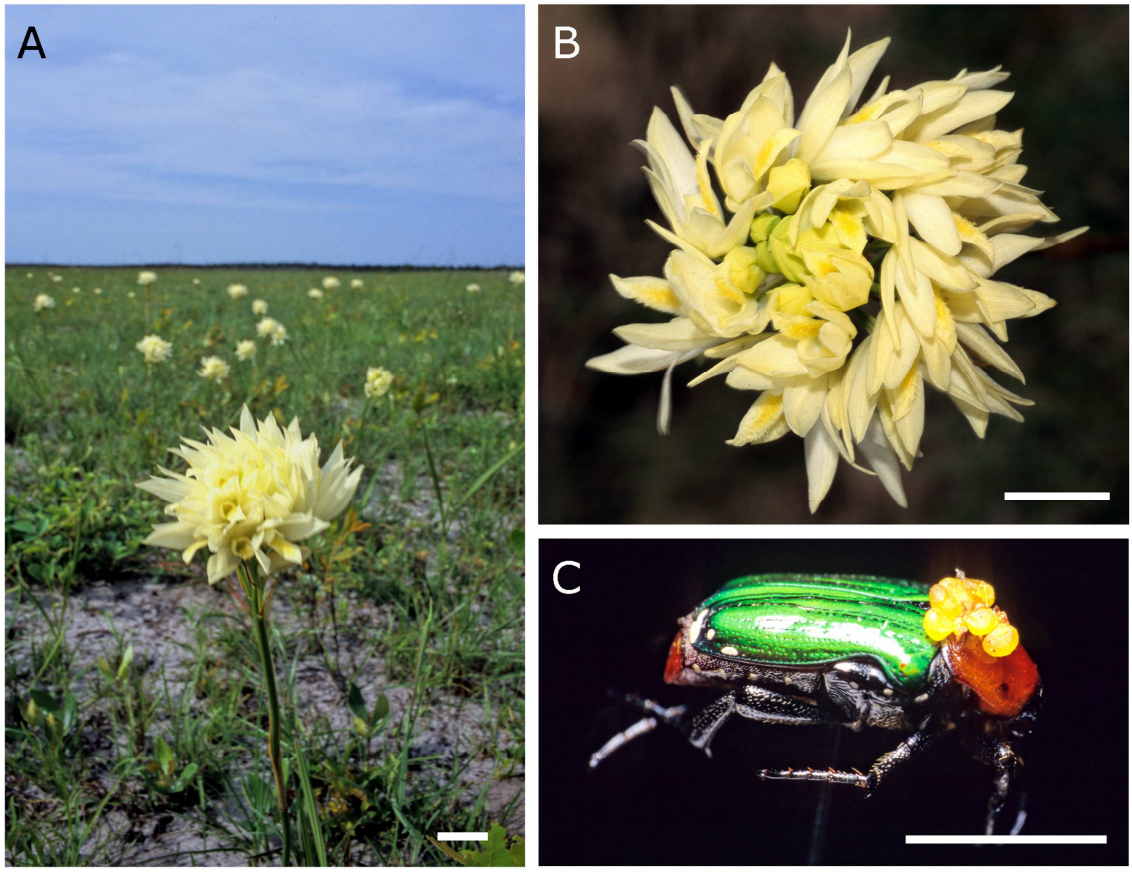


Figure 7: The cream form of *E. ensata* is often found in large populations (A) and is identical morphologically to the yellow form (Fig. 6), having dense, head-like inflorescences (B). C) This form is pollinated by *Leucocelis cf. amethystina*. Bar: A & B = 2 cm, C = 5 mm.

Eulophia welwitschia is apparently pollinated primarily by Cetoniid beetles which are larger than those that pollinate *E. ensata* (Table 2). In the southern Drakensberg and KwaZulu-Natal midlands *Atrichelaphinis tigrina* beetles were frequently found visiting the inflorescences of *E. welwitschia* and in a number of instances these beetles also carried pollinaria or viscidia (Fig. 8E).

Plants growing in Pretoria were pollinated by *Leucocelis* cf. *amethystina* beetles (Fig. 8F). These beetles are slightly smaller than the *Atrichelaphinis tigrina* beetles collected pollinating *E. welwitschia* in the Southern Drakensberg, however they are bigger than the *L. amethystina* beetles collected in KwaZulu-Natal. *L. amethystina* beetles were occasionally found in the vicinity of the orchids in the southern Drakensberg, but none of these carried pollinaria.

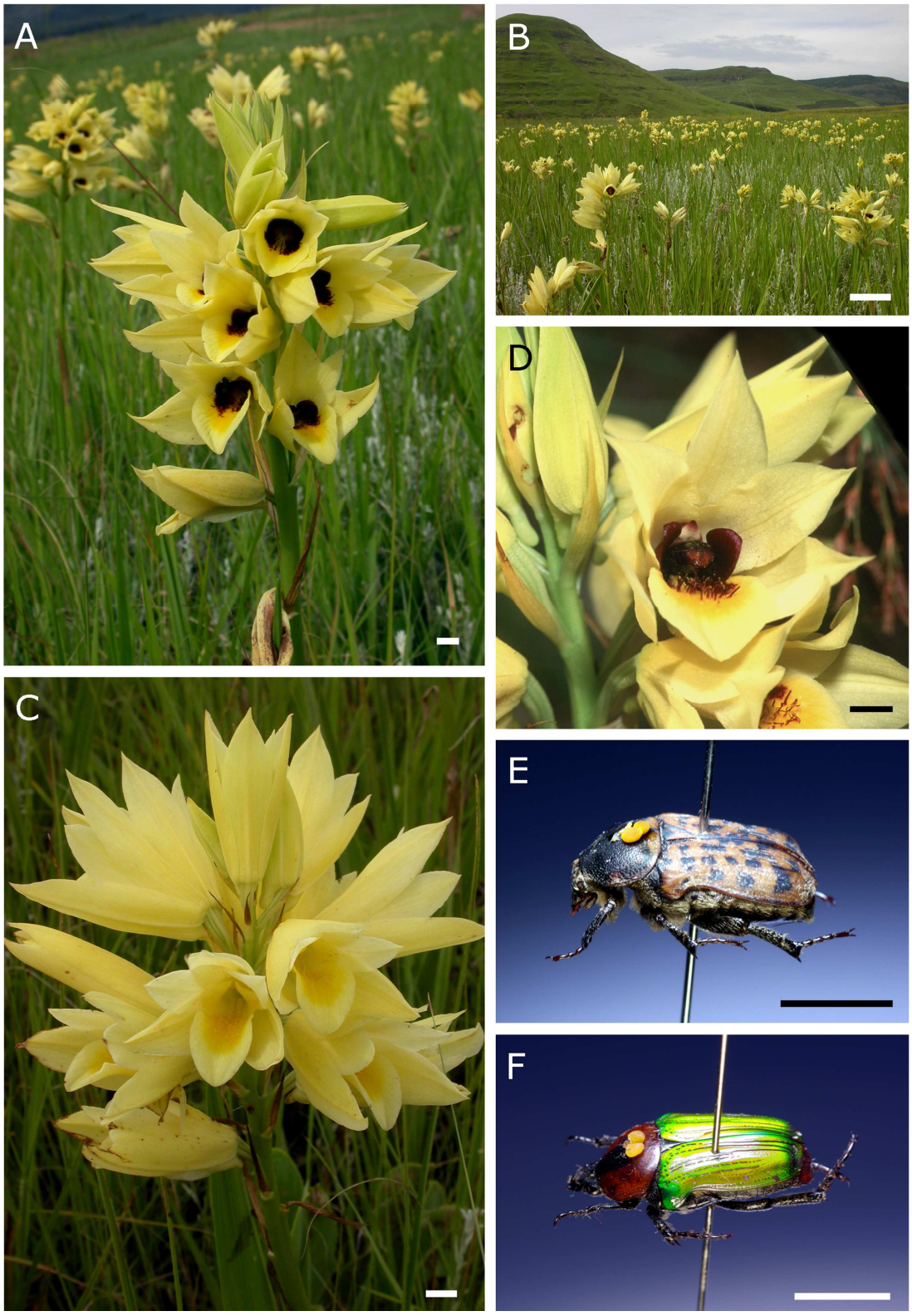


Figure 8: *Eulophia welwitschia* has butter yellow tepals and a distinctive dark maroon to black base of the labellum (A). This species often occurs in very large populations (B). In a number of populations in the southern Drakensberg, individuals lacking the dark labellum have also been observed (C). *E. welwitschia* is pollinated by Cetoniid beetles (D, E and F) including *Atricelaphinis tigrina* (E) and *Leucocelis* cf. *amethystine* (F). Bars: B = 5 cm, others = 5 mm.

As was also observed in *E. ensata*, two large halictid bees were found in the flowers of *E. welwitschia*, but these bees were sleeping in the flowers during rainy weather. One of these bees was kept alive in a vial with a flower and deposited the pollinia it was carrying on the stigma of the flower.

Anther cap retention also occurs in this species (average anther cap retention time 118 seconds, range 10 to 253 seconds). However the bending reconfiguration is never completed by the time the anther cap is dropped. Bending reconfiguration takes on average 223 seconds with a range of 98 to 450 seconds and is therefore the primary reconfiguration mechanism in this species.

Visitation rates and PTE

The rate of visitation to the deceptive flowers of these species is expected to be low and indeed very few direct visits by pollinators to flowers of the various species covered in this thesis were observed. Pollen transfer efficiencies are highest in the rewarding *Acrolophia cochlearis* and lowest in the species pollinated by small solitary bees (*E. ovalis*, long-spurred *E. parviflora* and perhaps *A. capensis*). Other species pollinated by larger bees and cetoniid beetles have relatively high rates of PTE ranging between 13 and 25% (Table 3).

Species where PTE has been examined in a number of populations show that PTE is particularly variable. For example, in *E. speciosa* PTE range from 0% for a road side population growing at Kenton-on-Sea in the Eastern Cape to 45.5% for a large population growing in the Bayhead Natural Heritage Site in the industrial area of Durban Harbour, KwaZulu-Natal (Table 3).

Table 3: Observed pollen transfer efficiencies in the different study species. Values in bold are means.

Date	Locality	n - inflorescences	n - flowers	% flowers with removal	% flowers pollinated	% Visited	% Failure	PTE %
E. speciosa (xylocopid bees[1])								
01 Nov 2001	Bayhead	30	210	5.2	2.9	12.4	53.8[2]	45.5
27 Dec 2001	Kariega	15	96	3.1	0.0	3.1	n/r[3]	0.0
22 Nov 2001	Bayhead	11	78	3.8	1.3	9.0	57.1[2]	16.7
29 Nov 2003	Kenton	15	107	8.4	1.9	9.3	n/r	11.1
07 Dec 2003	PA	13	117	12.0	6.0	12.8	6.7	25.0
07 Dec 2003	Kenton	7	67	11.9	3.0	16.4	27.3	12.5
13 Dec 2006	Kariega	10	43	39.5	14.0	46.5	15.0	23.5
13 Dec 2006	PA	14	77	16.9	2.6	16.9	0.0	11.5
30 Dec 2006	Kenton	19	132	22.7	6.1	25.8	11.8	13.3
				13.7	***4.2***	***16.9***	***24.5***	***17.7***
E. streptopetala (megachilid bees[1])								
07 Dec 2000	Nelspruit	26	200	22.0	4.5	22.5	2.2	10.2
28 Nov 2001	Umkomaas Valley	1	10	30.0	10.0	30.0	n/r	16.7
				26.0	***7.3***	***26.3***	***2.2***	***13.4***
E. cucullata (xylocopid bees[1])								
02 Nov 2001	Amatikulu	21	99	55.6	25.3	57.6	1.8	23.6
06 Nov 2001	Amatikulu	41	232	86.2	37.5	74.1	2.9	25.8
				70.9	***31.4***	***65.9***	***2.3***	***24.7***
E. angolensis (xylocopid bees) [1]								
13 Dec 2001	Mpenjati	5	89	21.1	7.9	25.8	4.3	23.7
15 Dec 2001	Mpenjati	17	287	27.2	7.0	31.0	10.1	16.7
05 Feb 2001	Mpenjati	23	446	7.0	2.0	7.6	n/r	14.5
				17.1	***4.5***	***19.3***	***10.1***	***15.6***
E. ovalis (halictid bees[1])								
06 Feb 2002	Cobham	23	106	42.5	5.7	50.0	13.2	8.9
E. zeyheriana (halictid bees[1])								
11 Feb 2002	Cobham	28	169	78.1	48.5	85.2	2.1	35.2
11 Dec 2002	Sani Pass	17	58	24.1	8.6	31.0	11.1	28.6
12 Dec 2002	Garden Castle	25	89	31.5	5.6	34.8	9.7	14.3
12 Dec 2002	Garden Castle	25	89	31.5	5.6	34.8	9.7	14.3
24 Jan 2003	Sani Pass	11	79	31.6	2.5	36.7	10.3	4.0
14 Jan 2004	Gilboa	149	641	33.4	11.5	39.8	13.7	28.0
23 Jan 2004	Gilboa	28	80	35.0	12.5	38.8	3.2	32.1
23 Jan 2004	Gilboa	80	407	24.1	8.6	25.6	3.8	29.1
				36.2	***12.9***	***40.8***	***8.0***	***23.2***
E. parviflora (long spurred form; anthophorid bees[1])								
12 Aug 2001	Krantzkloof	28	102	49.0	8.8	54.9	7.1	9.0
13 Aug 2001	Krantzkloof	36	174	17.2	3.4	21.3	16.2	10.0
19 Aug 2001	Stockville	24	97	11.3	0.0	14.4	21.4	0.0
24 Aug 2001	Krantzkloof	7	31	35.5	6.5	38.7	8.3	9.1
06 Aug 2002	Umtamvuna	6	28	10.7	0.0	10.7	0.0	0.0
10 Aug 2002	Stockville	26	117	12.8	2.6	13.7	0.0	13.3
12 Aug 2002	Stockville	34	213	9.9	1.4	11.7	16.0	7.1
19 Aug 2002	Stockville	13	55	9.1	1.8	9.1	0.0	10.0
27 Aug 2002	VCC transplant	22	101	18.8	2.0	21.8	9.1	10.5
27 Aug 2002	Stockville	19	94	13.8	0.0	17.0	18.8	0.0

16 Sep 2002	Krantzkloof	8	28	14.3	0.0	17.9	20.0	0.0
				18.4	***2.4***	***21.0***	***10.6***	***6.3***
Acrolophia cochlearis (colletid bees[1])								
Dec 2003	Grahamstown	20	184	70.7	47.3	82.6	3.3	38.5
2006/2007 season (average)[4]	Grahamstown	118	118	24.5	13.9	33.3	16.3	39.2
				47.6	***30.6***	***58.0***	***9.8***	***38.8***
E. ensata cream form; cetoniid beetles[1])								
30 Oct 01	Manzengwenya	20	205	3.4	2.0	3.4	n/r	28.6
15 Nov 01	Lake Sibaya	29	300	44.0	12.0	44.0	n/r	17.4
				23.7	***7.0***	***23.7***	***n/r***	***23.0***
E. ensata (yellow form; cetoniid beetles[1])								
16 Jan 03	Vernon Crookes	5	115	47.8	14.8	50.4	5.2	16.4
15 Jan 03	Thornville	16	414	46.2	14.3	45.1	1.2	17.2
				47.0	***14.6***	***47.8***	***3.2***	***16.8***
E. parviflora (short spurred form; cetoniid beetles[1])								
13 Oct 2000	VCC	27	262	69.5	30.5	73.3	n/r	22.0
16 Oct 2000	Balgowan	5	50	56.0	16.0	60.0	n/r	14.3
23 Oct 2000	Umgeni Valley	4	36	61.1	8.3	61.1	n/r	6.8
16 Sep 2002	Krantzkloof	7	43	55.8	20.9	58.1	4.0	37.5
02 Oct 2002	VCC	9	65	44.6	21.5	46.2	3.3	36.2
16 Oct 2002	VCC	24	138	52.2	18.8	57.2	7.6	20.1
08 Nov 2006	Grahamstown[5]	98	98	14.3	5.1	23.5	34.8	32.1
08 Nov 2006	Grahamstown[5]	11	70	57.1	7.1	68.6	14.6	10.0
				51.3	***16.1***	***56.0***	***12.9***	***22.4***
Eulophia petersii (pollinator Unknown)								
06 Dec 2000	Nelspruit	10	77	9.1	5.2	13.0	20	28.6
E. a. aculeata (pollinator unknown)								
29 Nov 2000	Grahamstown	8	63	66.7	20.6	71.4	6.7	25
Acrolophia capensis[2] (pollinator unknown)								
01 Dec 2003	Grahamstown	11	144	12.5	0.7	14.6	14.3	5.6
E. cristata (xylocopid bees)[6]								
1970s	Ghana	10	203	32.5	2.0	33.0	n/r	6.1

[1] Pollinators observed as part of this research.
[2] Failure rates at this site might be overestimated as a result of the activity of florivorous beetles.
[3] Failure rate not recorded.
[4] This value represents an average. See [chapter 5] for details.
[5] Two different populations one large and dense, the second small and sparse.
[6] Data from Lock & Profita (1975).

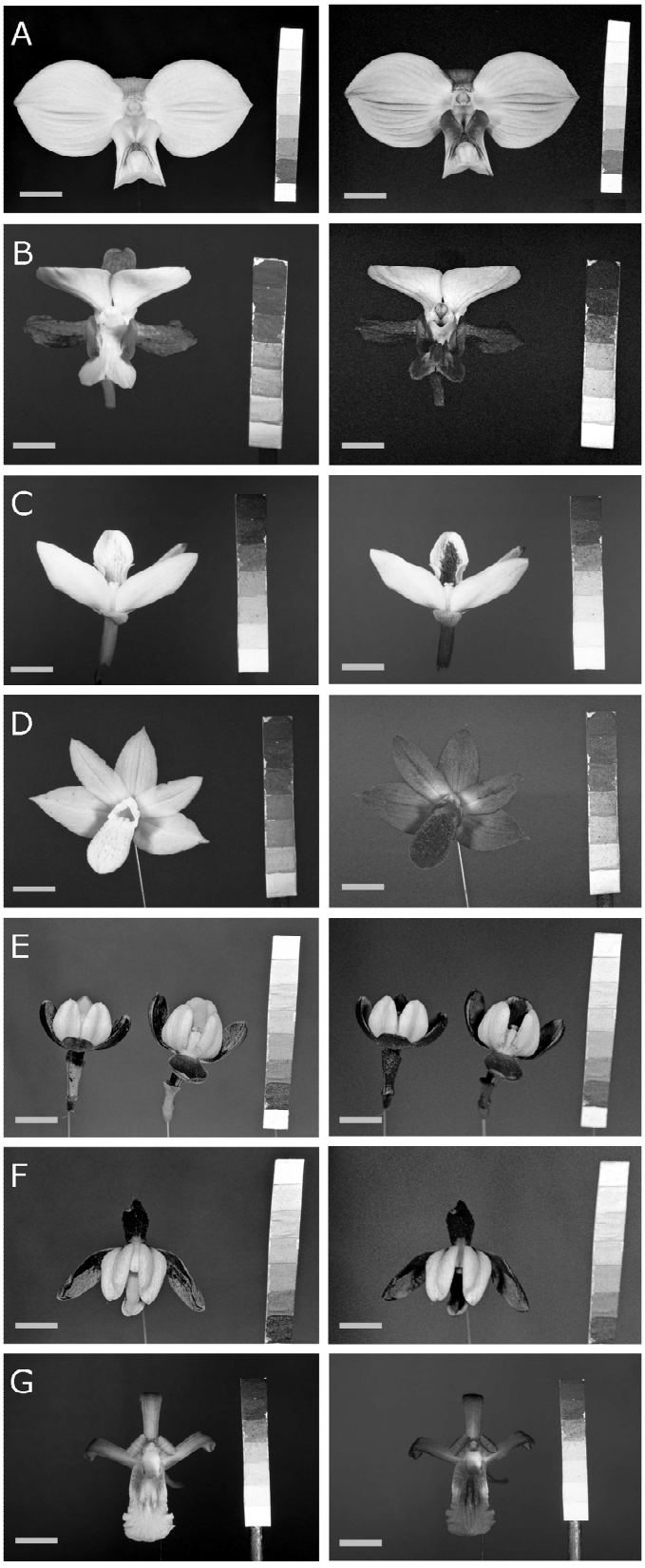


Figure 10:Human visual spectrum (400 to 700 nm; left column) and near ultra-violet (~360 nm to 400 nm; right column) images of flowers of A) *Eulophia speciosa*, B) *E. streptopetala*, C) *E. ovalis*, D) *E. ensata*, E) *E. parviflora* (short-spurred form), F) *E. parviflora* (long-spurred form), G) *E. petersii*. The lateral petals and labellum of all species besides *E. petersii* (green and brown) are yellow in the human visual spectrum. Bar = 5 mm.

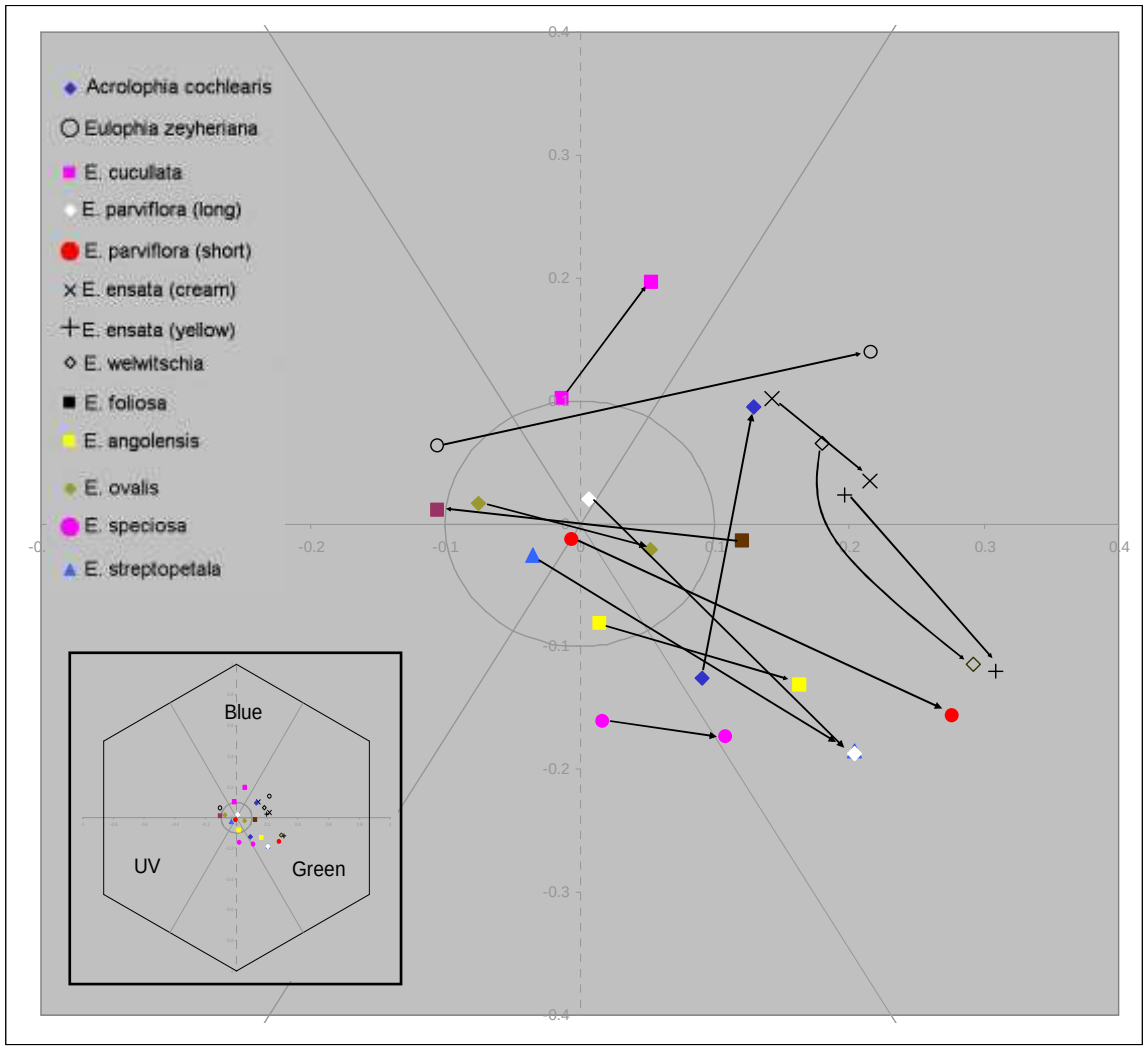


Figure 11: Comparisons of adaxial petal colour (start of arrow) and adaxial labellum colour (end of arrow) for a variety of *Eulophia* species for which the pollinators are known.

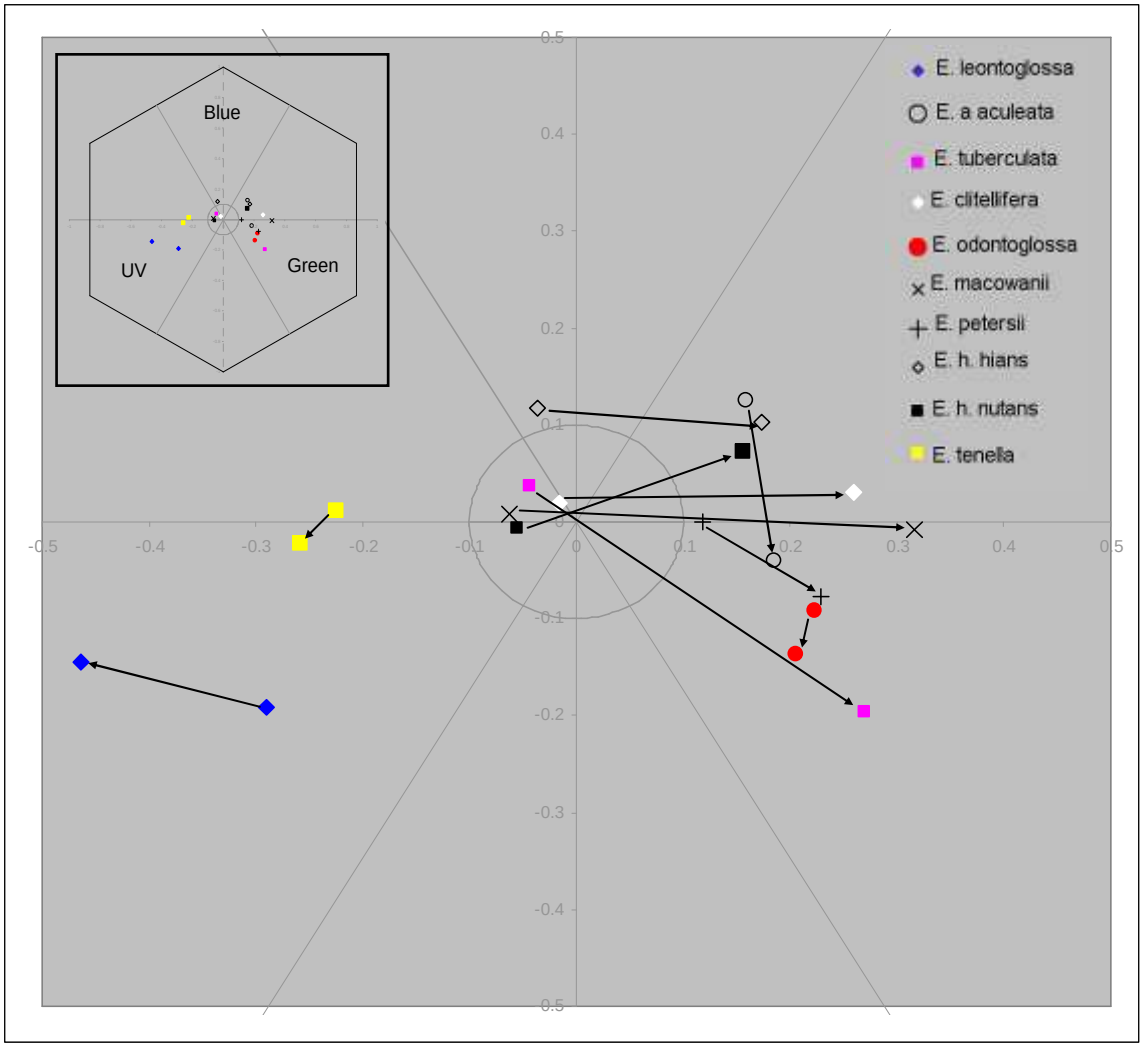


Figure 12: Comparisons of adaxial petal colour (start of arrow) and adaxial labellum colour (end of arrow) for additional species of *Eulophia* where the pollinators are unknown.

Table 4: Results of an experiment to determine the breeding system of *E. speciosa*. Test statistics apply to comparison of self- and cross-pollinated treatments.

	Unmanipulated Control n = 31	Cross-pollinated n = 10	Self-pollinated n = 11	Test statistic
Fruit set (%)	0	90	73	$G = 0.12^{ns}$
Mean capsule & seed mass in grams ($\bar{x} \pm se$)	-	0.90 ± 0.15	0.64 ± 0.12	$t_{19} = 1.31^{ns}$
Mean seed mass in grams ($\bar{x} \pm se$)	-	0.27 ± 0.07	0.14 ± 0.02	$t_{19} = 1.28^{ns}$
Percent seeds with embryos ($\bar{x} \pm se$)	-	78.6 ± 3.9	39.7 ± 6.6	$t_{15} = 4.58^{***}$

Table 5: Results of an experiment to determine the breeding system of *E. angolensis*. Results show percentage or mean ± se. Test statistics apply to comparison of self- and cross-pollinated treatments.

	Unmainpulated Control	Cross-pollinated	Self-pollinated	Test statistic	Naturally pollinated flowers
Percent fruit set (n)	0 (20)	83 (18)	82 (17)	$G = 0.001^{ns}$	n/a^2 (24)
Mean capsule & seed mass in grams [$\bar{x} \pm se$ (n)]	-	0.64 ± 0.06 (15)	0.68 ± 0.07 (14)	$t_{33} = 0.3^{ns}$	0.67 (24)
Mean seed mass in grams [$\bar{x} \pm se$ (n)]	-	0.18 ± 0.03 (15)	0.19 ± 0.04 (14)	$t_{33} = 0.1^{ns}$	0.19 (24)
Percent seeds with embryos [$\bar{x} \pm se$ (n)]	-	16.1 ± 1.2 (15)	9.8 ± 1.9 (14)	$t_{26} = 3.3^{**}$	17 (21)

Table 6: Results of an experiment to determine the breeding system of *E. welwitschia*. Results show percentage or mean ± se. Test statistics apply to comparison of self- and cross-pollinated treatments.

	Unmanipulated Control	Cross-pollinated	Self-pollinated	Test statistic
Percent fruit set (n)	0 (29)	94 (18)	100 (19)	$G = 0.03^{ns}$
Mean capsule & seed mass wet weight in grams [$\bar{x} \pm se$ (n)]	-	2.43 ± 0.31 (18)	2.85 ± 0.21 (19)	$t_{35} = 1.12^{ns}$
Mean capsule dry weight in grams [$\bar{x} \pm se$ (n)]	-	0.41 ± 0.05 (18)	0.50 ± 0.04 (17)	$t_{35} = 1.42^{ns}$

Flower colour

The species of *Eulophia* examined have a diverse range of floral colours, with pink and yellow being common (see various colour plates in this thesis). Colours also frequently include an obvious UV component (Fig. 12).

Perhaps the most striking aspect of flower colour in most of the species examined is the contrast between the colours of the lateral petals and labellum. In many cases the colour of the central labellum lobe is shifted to the lower left of the colour hexagon corresponding to bee green (yellow, UV absorbing; Figs. 10 & 11). This pattern is easily visualised through UV photography and Fig. 12 shows the contrast between the light UV reflecting surfaces of the petals and the darker UV absorbent surface of the central lobe of the labellum for a number of species. To humans, most of the petals and labellae in Fig. 12 appear yellow.

Breeding systems

There is little difference in the overall quantity of fruit resulting from cross- and self-pollination in *E. speciosa*, *E. angolensis* and *E. welwitschia* (Tables 4 - 6). However in *E. speciosa* and *E. angolensis* the quality of seeds produced from cross-pollinated capsules is significantly higher than that produced from self-pollination. There is no evidence of auto-pollination in these three species

DISCUSSION

The data presented here show that many *Eulophia* species are specialized for pollination by bees. Large and showy species such as *E. speciosa*, *E. streptopetala*, *E. cucullata* and *E. angolensis*, as well as *E. cristata* (Lock & Profita 1975) are pollinated by medium to large *Xylocopa* and megachilid bees. The observations that *E. speciosa* is pollinated by the large carpenter bee *Xylocopa flavorufa* are congruent with those of van der Cingel (2001) who observed a large xylocopid bee visiting *E. speciosa* on Inhaca island in southern Mozambique. There is a high probability that the bee van der Cingel (2001) observed was also *X. flavorufa* as there are only two very large *Xylocopa* bees in southern Africa and *X. flavorufa* is by far the most common in coastal habitats. Similarly the observations of Dino Martins (Martins 2002; pers. com.) in Tanzania of very large *Xylocopa nigrita* bees visiting and removing the pollinaria of the very large, pink flowers of *E. horsfallii* on the posterior margin of the metathorax, support the preliminary observations of Kullenberg (1961) for this species.

The traits of the *Xylocopa*-pollinated species are similar to those of other carpenter bee-pollinated Angiosperms. These include: large flowers, spacious enough to admit these large bees; strong, heavy tissued flowers, robust enough to withstand (in the quaint words of van der Pijl) "rude insects and their rough foot work"; "well hidden nectar protected by closing mechanisms … to exclude unwanted visitors"; sexual organs brought into contact with the dorsal part of the insect (van der Pijl 1954a, p 423); with pale unsaturated colours and "fresh" scents (van der Pijl 1954b, p 559). Obviously these deceptive orchids do not possess any nectar rewards, but some species (*E. speciosa* and *E. streptopetala*) have spurs or folds that the bees attempt to manipulate to access a reward. The colours of the *Xylocopa*-pollinated *Eulophia* species are either bright yellow or bright pink. These two colours are common in other *Xylocopa*-pollinated plants in South Africa (pers. obs.), India (Solomon Raju & Purnachandra Rao 2006) and the tropics (van der Pijl 1954a, van der Pijl 1954b, van der Pijl 1954c), van der Pijl's (1954b) generalisation of unsaturated colours notwithstanding. Scents to the human nose are variable, ranging from odourless or very faintly scented (*E. speciosa* and *E. streptopetala*), to strongly and pleasantly scented (*E. angolensis*), to the unusual 'chemical' scent of *E. cucullata.*

Interestingly *E. cucullata* and *E. angolensis* not only share *Xylocopa flavicollis* as a pollinator, but also have a common point of pollinarium attachment on the dorsal, posterior margin of the metathoracic segment between the wings. In addition these two species have similar distributions and potentially similar habitat requirements with *E. angolensis* being found in moist grasslands and swamps while *E. cucullata* is common in moist grasslands surrounding such swamps. As a result it will be interesting to explore more northerly populations for evidence of hybridization. I performed a preliminary experimental cross between these two species and this resulted in a fruit with 79% of the seeds having embryos, suggesting that these two species are inter-fertile.

The fact that species such as *E. ovalis*, *E. zeyheriana* (Peter & Johnson 2008 [chapter 2]) and the long-spurred form of *E. parviflora* [chapter 6] with relatively small flowers and lax inflorescences are pollinated by small halictid or anthophorid bees also supports the initial hypothesis of bee-pollination. Inflorescences of these species are few-flowered, have spurs longer than the tongues of the insects and with the exception of the long-spurred form of *E. parviflora*, are apparently scentless, at least to the human nose.

In contrast to these bee-pollinated species, the presence of beetle-pollination in a number of species (*E. ensata*, *E. welwitschia*, *E. foliosa* [chapter 3] and the short-spurred form of *E. parviflora* [chapter 6]) was unexpected and at odds with the initial hypothesis of widespread bee pollination in the genus. However, this is due to the initial failure to recognise the importance of dense, crowded inflorescences as a trait associated with beetle-pollination (cf. van der Pijl 1961, Faegri & van der Pijl 1979). All the beetle-pollinated *Eulophia* species examined here have dense, crowded inflorescences that approximate a course brush inflorescence (cf. Bernhardt 2000).

The bright yellow and cream-flowered *Eulophia* species with dense inflorescences are similar to those of *Ceratandra grandiflora,* a deceptive orchid which is pollinated by monkey beetles (Steiner 1998). In this species, the beetles are thought to aggregate on the deceptive inflorescences which serve as a rendezvous site for mating purposes. It is possible that similar rendezvous behaviour occurs on these deceptive *Eulophia* species (*E. ensata*, *E. welwitschia*, and the short-spurred form of *E. parviflora* [chapter 6]) as the cetoniid beetles spend some time clambering around the inflorescences but do not seem to systematically probe all the flowers in search of a food reward. As noted by Peter & Johnson (2006a [chapter 3]), *E. foliosa* also has dense inflorescences with similarities to some of the northern hemisphere generalist species such as *Listera ovata* and *Dactylorhiza* (*Coeloglossum*) *viridis*, which are frequented by various beetles (amongst a number of other insect orders), the South African species *Satyrium microrrhynchum* pollinated by pompilid wasps and the cetoniid beetle *Atrichelaphinus tigrina* (Johnson *et al.* 2007), and the specialised South American orchid *Pteroglossaspis ruwenzoriensis* with jelly-like nectar pollinated exclusively by the cetoniid beetle *Euphora lurida* (Singer & Cocucci 1997). The noticeable difference between these pale green flowered orchids with dense inflorescences and *E. foliosa* is that the latter is deceptive.

The crowded inflorescences of some species may be described as capitate and in *E. ensata* approximate the flat-topped inflorescence of co-occurring Asteraceae such as various species of *Helichrysum*. It is possible therefore that *E. ensata* mimics these rewarding plants. The fact that flowers of *E. ensata* are entirely UV absorbing unlike many other yellow *Eulophia* species where the petals reflect UV light (Fig. 10) supports this hypothesis as the measured reflectance spectra of the inflorescences of species such as *Helichrysum nudifolium* are strongly UV absorbent (data not shown) possibly signalling the presence of abundant pollen rewards to pollen-feeding beetles and bees (cf. Heuschen *et al.* 2005). Interestingly, the flowers of the monkey beetle-pollinated *Ceratandra grandiflora* are yellow and absorb UV although, as noted

above, this species is thought to be pollinated by rendezvous pollination and not food mimicry (Steiner 1998). If *E. ensata* does indeed represent a case of specific mimicry of rewarding *Helichrysum* inflorescences, then the unique inflorescence architecture of this species is comparable to the capitate inflorescence of *Disa cephalotes* subspecies *cephalotes* and *Brownleea galpinii* subspecies *major* that are adapted to exploit the relationship between long-tongued flies and the rewarding capitate inflorescences of *Scabiosa columbaria* (Johnson *et al.* 2003a).

There is some uncertainty as to the role of bees that have been seen visiting both of the putative beetle-pollinated species described here (*E. ensata* and *E. welwitschia*). If these species are indeed specialised mimics of asteraceous inflorescences as discussed above, it is possible that solitary bees might also contribute to the pollination of these deceptive orchids as these bees frequent the "model" inflorescences. In addition, the dark centre of the *E. welwitschia* was initially thought to attract roosting bees as has been noted for a few Mediterranean orchids and irises (cf. Pellegrino *et al.* 2005, Sapir *et al.* 2005). However the slow pollinarium reconfiguration in both these species is not consistent with rapid visits by solitary bees and supports the contention that these two species are indeed adapted for beetle pollination having comparable reconfiguration times to those of the beetle-pollinated short-spurred form of *E. parviflora* [chapter 6]. The slow reconfiguration of the pollinaria might therefore represent adaptations to the most effective pollinators which in this case are slow moving cetoniid beetles.

The specialised pollen mimicry suggested above and the case of specific pollen mimicry in *E. zeyheriana* proposed by Peter and Johnson (2008 [chapter 2]) are distinct from the more generalised pollen signalling found in many other flowering species such as the Asteraceae (Heuschen *et al.* 2005) and Aizoaceae (Peter *et al.* 2004). Heuschen *et al.* (2005) suggest that the occurrence of uniform yellow, UV absorbent centres in many unrelated lineages of Angiosperms represents an unrecognised form of generalised mimicry. In many cases however these markings, in the centre of flowers, are in close association with the anthers bearing the pollen which serves as a reward to the pollinators and so the yellow UV absorbing colours of the petals are a legitimate signal to pollinators.

The system of "generalised" pollen mimicry in these deceptive orchids proposed here is therefore quite distinct from the generalised pollen mimicry proposed by Heuschen *et al.* (2005). The flowers have no reward, but the textured labellae of a number of species are

distinct from that of the laterals petals (and coloured sepals in some species) falling in the bee green region of the colour hexagon. This corresponds to a general yellow, UV absorbent colour characteristic of pollen (Heuschen *et al.* 2005). Because these flowers have no reward, these "pollen patches" on the labellae may serve as generalised mimics of pollen or anthers in these species albeit without mimicking a specific model species.

Future flower painting experiments will test this generalised pollen mimicry hypothesis. By modifying the yellow, UV absorbing labellae of species such as the long-spurred form of *E. parviflora* and *E. angolensis* to be UV reflecting, it may be possible to establish if these pollen mimicking labellae are indeed important signals to the pollinators.

Pollinarium reconfiguration

This study identifies three modes of pollinarium reconfiguration in *Eulophia.* This includes two modes of pollinarium bending, the *E. speciosa*-type reconfiguration found in a few species and the more common *E. streptopetala*-type reconfiguration (Fig. 9). Two of the species examined possess an unusual mode of reconfiguration – anther cap retention which is explained in detail by Peter & Johnson (2006a [chapter 3]). Authors such as Darwin (1867) and van der Pijl & Dodson (1966) describe a number of possible reconfiguration modes that may not be homologous with those described here. A detailed survey of pollinarium reconfiguration in the orchidaceae is required to make sense of this variation.

Pollen transfer efficiency

Rates of pollen transfer efficiency (PTE) along with other measures of visitation such as pollinarium removal and pollinia deposition in *Eulophia* are variable both within and between species (Table 3). The highest PTE values were recorded in the rewarding *Acrolophia cochlearis* and this is discussed further in chapter 5.

The species with the lowest PTE scores include the two species (*E. ovalis* and the long-spurred form of *E. parviflora*) pollinated by solitary bees and which are thought to employ generalized food deception. These species have rates of pollinarium removal comparable to many of the other species analysed, but very low rates of pollinia deposition. This suggests that plants with these systems have inefficient deposition rates on stigmas or that the insects rapidly learn to avoid these rewardless species. Although the pollinator for *Acrolophia capensis* has not been identified, this species has many characteristics in common with these two *Eulophia* species pollinated by small solitary bees.

In contrast the batesian mimic *E. zeyheriana* gets subsidised by positive conditioning of the solitary *Lipotriches* bees by the rewarding model species *W. cuspidata* (Peter & Johnson 2008 [chapter 2]) resulting in relatively high rates of PTE (Table 3). The other generalized food deceptive species pollinated by larger bees such as *Xylocopa* and *Megachile* have surprisingly high PTEs.

Breeding System

Tremblay (2005) has reviewed, amongst other aspects of orchid reproductive biology, the breeding systems of orchids. This work suggests that in many species similar levels of fruit set can be expected in self- and cross-pollinated flowers. However the quality of these fruit tends to differ, with capsules resulting from cross-pollination having a third more fertile seeds than self-pollinated flowers (also reviewed by Jersáková *et al.* 2006).

The xenogamous species of *Eulophia* and *Acrolophia* examined to date follow this pattern. Comparable levels of fruit set can be expected from self- and cross-pollinated flowers (both in terms of percentage fruit set and the mass of the capsules), however the quality of seed produced by cross-pollinated flowers is significantly higher in all species examined. These observations hold for *E. speciosa* (Table 4), *E. angolensis* (Table 5), *E. zeyheriana* (Table E1 of chapter 2), *E. foliosa* (Table 3 of chapter 3). It is surprising that orchid breeding systems presenting only data on the quality of capsules and not the percentage of fertile seeds are still being published.

Pollination in other species of Eulophia

Using data presented here, it is possible to speculate on the pollination systems of other common South African *Eulophia* species (Table 7). For example *E. tuberculata* and *E. clitellifera* (Fig. 13 A and B) have very similar floral (and vegetative morphology) to *E. speciosa* and these taxa are undoubtedly closely related. Given these similarities of spreading lateral petals, short fold-like spur, short stout column and pollinaria with the same *E. speciosa*-type reconfiguration, it is reasonable to expect head placement of pollinaria on small to medium anthophorid bees in species such as *E. tuberculata* and *E. clitelifera.*

Similarly, thoracic pollinarium placement on large to very large Xylocopid bees can be expected in the large pink and yellow flowered species found in tropical Africa. These include species such as *E. horsfallii* (Fig. 13C), *E. livingstoniana*, *E. latilabris* and *E. coeloglossa.*

E. petersii (Fig. 13D) and *E. leachii* can be predicted to employ thoracic pollinarium placement given the tubular inner flower structure similar to that of *E. angolensis*. However the rather drab flower colour seems at odds with *Xylocopa* pollination.

Species with dense, capitate inflorescences are likely to be beetle-pollinated species and the two colour forms of *E. leontoglossa* (Fig. 13H & I) as well as the two subspecies of *E. aculeata* (Fig. 13F & G) fit this criterion. However they differ from the beetle-pollinated species examined to date by having pendant flowers making up the crowded inflorescence.

A few tropical African species do not conform to any of these predictions. For example *E. walleri* has unusual floral morphology and orange-red flowers suggesting bird or butterfly pollination while *E. guineensis* has a large, prominent white labellum and a long slender spur reaching 20 mm hinting at the possibility of moth pollination. I know of no deceptive moth pollinated species and it will be interesting to determine if this species is rewarding given that no rewarding species of *Eulophia* have been discovered to date.

In conclusion, the results of this chapter and others in the thesis contribute to an understanding of the pollination biology of a number of species of this important African orchid genus, and makes predictions about the pollination of other species. Future studies should focus on the pollination of *Eulophia* species in south-central Africa, as this is the centre for diversification of the genus

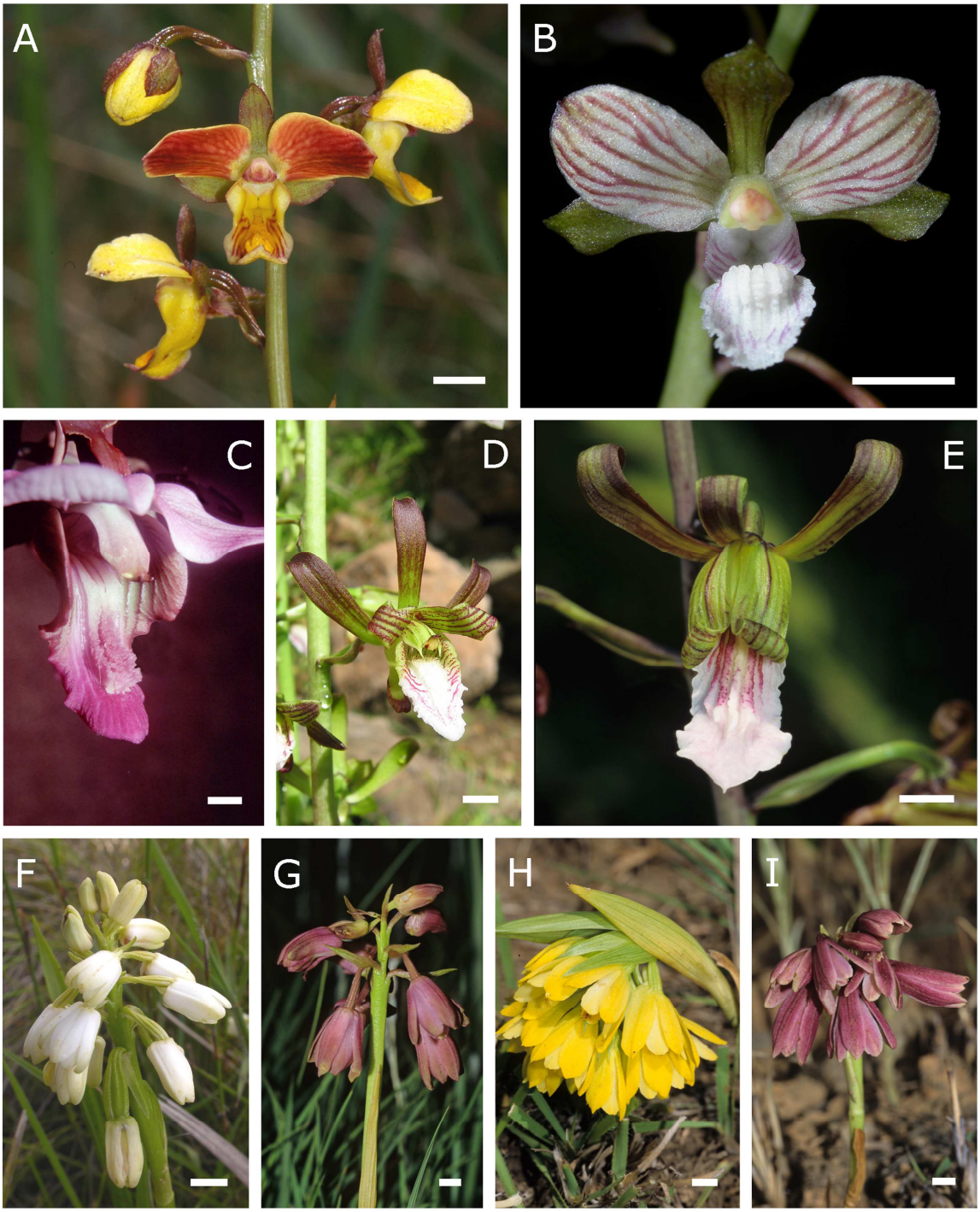


Figure 13: Other potentially bee- and beetle-pollinated species of *Eulophia*. *Eulophia tuberculata* (A) and *E. clitellifera* (B) have similar floral characteristics to *E. speciosa* and head placement of pollinaria can be expected on small bees. Visits to *E. horsfallii* (C) by very large *Xylocopa nigrita* carpenter bees in Tanzania have been observed by Dino Martins (per. comm.). In the case of the "tubular" flowers of *E. leachii* (D) and *E. petersii* (E), thoracic placement of pollinia on larger Xylocopid bees is likely. While the dense capitate inflorescences of *E. aculeata* subspecies *aculeata* (F), *E. a.* subspecies *hutonii* (G) and the two colour forms of *E. leontoglossa* (H and I) is consistent with beetle pollination, the pendant arrangement of flowers in these species is not. Bar = 5 mm.

Table 7: Predictions of pollinators for other species of *Eulophia* based on traits described in the current study.

Traits	Likely pollinators	*Eulophia* examples [chapter]	Predicted *Eulophia* species	Uncertainties
Short gynostemium; relatively large and exposed stigma; spreading lateral petals; short fold-like spur; *E. speciosa*-type reconfiguration.	Small to medium anthophorid bees, head placement of pollinaria. *E. speciosa*-type mode of pollinarium reconfiguration.	*E. speciosa* [current]	*E. tuberculata* (Fig. 3A) *E. clitelifera* (Fig. 13B) *E. schweinfurthii* *E. meleagris* *E. fridericii*	*E. meleagris* is a forest margin species with dark purple flowers.
Crowded inflorescences; slow pollinarium reconfiguration mechanism.	Beetle, probably cetoniid beetles.	*E. foliosa* [3] *E. ensata* [current] *E. welwitschia* [current] *E. parviflora* short-spurred form [6]	*E. leontoglossa* (Figs. 13H & I) *E. aculeata* (Figs. 13F & G)	Pink and white colours not consistent with other beetle-pollinated *Eulophia* species. Flowers pendant.
Relatively large flowers, pale to bright purple and pink.	Medium to large xylocopid bees. Thoracic placement of pollinaria. *E. streptopetala*-type mode of pollinarium reconfiguration	*E. cucullata* [current] *E. horsfallii* (Dino Martins pers. com., Fig. 13C) *E. cristata* (Lock & Profita 1975)	*E. latilabris* *E. ceologlossa* *E. livingstoniana* *E. calantha*	
Petals and labellum form "tube" around the gynostemium.	Medium to small xylocopid bees. Thoracic placement of pollinaria. *E. streptopetala*-type mode of pollinarium reconfiguration	*E. angolensis* [current]	*E. petersii* (Fig. 13E) *E. leachii* (Fig. 13D)	Dull green and brown colours not consistent with xylocopid pollination.

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[chapter 8]

Autonomous self-pollination in South African species of *Eulophia*

ABSTRACT Autonomous self-pollination ("auto-pollination") is surprisingly common among orchids and is thought to provide reproductive assurance in the face of low rates of pollinator visitation. Consistently high rates of capsule set were observed in *E. clavicornis* Lindl. var. *clavicornis*, *E. c.* var. *inaequalis* (Schltr.) A. V. Hall, *E. c.* var. *nutans* (Sond.) A.V. Hall. and *E. tenella* Rchb. f. and experiments showed that these taxa are capable of auto-pollination. The mechanism was investigated using microscopy and this showed that the rostellum tissue is often interrupted or completely absent allowing the pollinia to come into contact with the stigmatic fluid resulting in germaination of the pollen tubes, ensuring pollination. There are a number of variations on this basic mechanism. In a number of instances, basal flowers on an inflorescence are apparently functional for insect pollination and possess an intact rostellum and functioning pollinaria. At the distal end of the inflorescence flowers often lack pollinia and do not set capsules. Emasculated flowers of *E. c.* var. *nutans* did not set fruit, suggesting that agamospermy is an unlikely explanation for the high levels of fruit production. Other *Eulophia* species, notably *E. speciosa* and *E. streptopetala* sometimes appear to have very high levels of fruit set. However, investigations showed that most of these "fruits" are the result of insect parasitism. Therefore, auto-pollination should not be inferred directly from apparent high levels of fruit set.

INTRODUCTION

Autonomous self-pollination ("auto-pollination", sensu Catling, 1990)[3] has been known in orchids since 1833 when Brown described the phenomenon in *Ophrys apifera* (Catling 1990). Darwin (1867) was troubled by the case of *O. apifera* and 23 other species of orchids known at that time to undergo auto-pollination, since he had gone to considerable effort to emphasize the adaptive significance of mechanisms such as pollinaria reconfiguration (Peter & Johnson 2006 [Chapter 4]) that prevent self-pollination in other orchids.

Darwin did, however, recognise that self-pollination including auto-pollination might be adaptive under conditions of pollinator and mate limitation (Darwin 1876, Lloyd 1992), but there is conflicting empirical evidence for this (Herlihy & Eckert 2002, Kalisz *et al.* 2004). Such conditions, particularly pollen limitation, are known to be frequent among deceptive orchids (Neiland & Wilcock 1998, Tremblay *et al.* 2005, Smithson 2006) and rewardless clades may thus be expected to have higher numbers of auto-pollinating species than their rewarding relatives, although I am not aware of any formal tests of this prediction.

Catling's (1990) review of the incidence of auto-pollination in the orchids showed that auto-pollination occurs in all orchid subfamilies and the majority of tribes and subtribes, with 350 species known at that time to possess mechanisms of auto-pollination. As described in chapter 1, I used the information in van der Cingel (1995, 2001), subsequent peer reviewed articles, and the list of auto-pollinating species compiled by Catling (1990), to derive an up-to-date estimation of the importance of auto-pollination in the Orchidaceae. This analysis suggests that auto-pollination is common in the family, making up 31% of the species with known pollination systems [Table 1 of chapter 1] and raises the number of auto-pollinating species to 395. Auto-pollinating species are therefore second only to the bee-pollinated species in terms of numbers in the family, although it is likely that this is a gross over representation given the relative ease of identifying auto-pollinating species.

[3] Catling (1990) points out that self-pollination without the action of a pollinator has been termed autonomous autogamy, autonomous self-pollination and spontaneous self-pollination. In place of these rather clumsy terms I have in the text adopted Catling's (1990) term "auto-pollination" which is a concatenation of autonomous self-pollination and accurately differentiates autonomous, vectorless pollination from self-pollination mediated by a pollinator (pollinator mediated autogamy or facilitated selfing). The use of the term "self-pollination" is equivocal. Self-pollination between different flowers on an individual is unambiguously known as geitonogamy. Cleistogamy is widely and consistently used to describe automatic self-pollination within unopened buds. However, self-pollination within a single flower either through the services of a pollinator or through the action of the floral organs is often referred to under the catch-all term "autogamy".

Auto-pollination in Eulophia *and related genera*

In the tribe Cymbidieae, auto-pollination has been recorded in a number of species including *Oeceoclades maculata* (Gonzalez & Ackerman 1988) and *Eulophia alata* (Catling 1990). In these species the stipe of the pollinarium bends sufficiently for the pollinia to be deposited over the rostellum and onto the stigma. In addition, Williamson (1984) documented auto-pollination as a result of the absence of rostellar tissue in eight *Eulophia* species from west Africa although two of these taxa were reduced to synonomy of a third by Thomas (1998).

While working on the pollination biology of the South African species of *Eulophia,* I have examined 29 taxa (mostly species but also including a number of subspecies or varieties as defined by Hall 1965) of which four taxa showed very high levels of fruit set across different sites and different years suggesting the possibility that these species might be auto-pollinating. I therefore set out to determine the occurrence and mechanism of auto-pollination in these taxa. I also examined several other species in which fruits appeared to develop without flowers being pollinated.

MATERIAL AND METHODS

The study taxa and sites

Three of the taxa examined are given varietal status by Hall (1965). These include *E. clavicornis* Lindl. var. *clavicornis* (Fig. 1a), *E. c.* var. *inaequalis* (Schltr.) A. V. Hall and *E. c.* var. *nutans* (Sond.) A.V. Hall (Fig. 1b). The fourth taxon is the species *E. tenella* Rchb. f. (Fig. 1c). In a number of cases, individuals of known out-crossing species were observed to develop capsules, seemingly without being pollinated. These include individuals of *E. speciosa*, *E. streptopetala*, *E. zeyheriana*, and *A. cochlearis*. Observations were conducted at a variety of sites as listed in Table 2.

Table 2: Sites at which the study taxa were examined.

Taxon	Sites[1]
E. clavicornis var. *clavicornis*	1, 2, 4, 18, 20, 31, 37, 38, 39
E. clavicornis var. *inaequalis*	6, near 31
E. clavicornis var. *nutans*	24, 25, 26, 28, 32
E. tenella	2, 13
E. speciosa	40
E. streptopetala	Bathurst State Forest near 2
E. zeyheriana	14, 25
A. cochlearis.	2

[2] Numbers correspond with those used in Map 1 & 2 at the end of this thesis.

Auto-pollination mechanisms

The gynostemia of fresh and preserved flowers were examined with a dissecting microscope and imaged digitally. Additional images were collected using scanning electron microscopy and macro photography using a bellows.

For each of the four auto-pollinating taxa as well as an auto-pollinating form of *E. zeyheriana*, the percentage of flowers in each of four categories (presence or absence or rostellum combined with presence or absence of pollinia – see Fig. 3) was determined using a dissecting microscope. Flowers were also scored for fruit set as, in all but the most recently opened flowers, it is possible to determine whether or not a capsule is being produced by the state of the ovary which swells rapidly following auto-pollination. A chi-square contingency test was used to compare the frequency of flowers setting fruit versus those that did not set fruit across each of the four categories.

Breeding systems and manipulations

Breeding system experiments were attempted for *E. c. clavicornis*, *E. c. nutans* and *E. tenella*. The flowers of bagged inflorescences were self-pollinated, cross-pollinated or left unmanipulated. In addition, some flowers were emasculated to test for agamospermy. In the case of *E. c. clavicornis* the vast majority of inflorescences were predated by lepidopteran larvae, while in the *E. tenella* experiment, a hot dry spell followed the experimental manipulations and all but one inflorescence wilted well before the capsules had matured.

Only the breeding system experiments for *E. c. nutans* was successful. Capsules produced were weighed, as was the mass of seeds produced. A sub-sample of seeds was examined under a dissecting microscope and the percentage of fertile and infertile seeds determined.

RESULTS

Occurrence

Only plants of *E. c. clavicornis*, *E. c. nutans*, *E. c. inaequalis* and *E. tenella* consistently possess an auto-pollination mechanism described below and show high rates of capsule set (Fig. 1). One individual of *E. zeyheriana* from Sani Pass in the Drakensberg and a small population of this species from Ugie in the southern Drakensberg also exhibit this auto-pollination mechanism.

The mechanism of auto-pollination

Eulophia clavicornis clavicornis, *E. c. nutans, E. c. inaequalis* and *E. tenella* as well as a few isolated individuals of *E. zeyheriana* all share a common mechanism of auto-pollination. Typically this entails the absence of the rostellum (the small flap of tissue separating the stigmatic cavity from the area under the anther cap housing the two pollinia) which fails to develop in many flowers. The absence of rostellar tissue allows the pollinia to come into contact with the stigmatic fluid, causing them to swell (often distorting the anther cap tissue) and the pollen tubes to grow *en masse* into the stigma. In most cases observed, the tissue of the anther cap is firmly attached to the tip of the gynostemium and it is unlikely that an insect visiting the flower would succeed in removing the pollinarium (Fig. 2).

There are a number of subtle variations on this basic mechanism. In *E. tenella* the lower flowers of many inflorescences appear functional, with an intact rostellum as well as functional pollinaria that in some cases can be removed from the flower although no evidence of pollinaria removal was seen in the numerous flowers inspected (Fig. 2a).

The majority of flowers fit the basic description of possessing a degenerate rostellum although the degree of degeneration varies from flower to flower. Lower flowers typically only have a small gap in the rostellum allowing only one of the paired pollinia to grow into the stigma (Fig. 2b). Some flowers have two gaps corresponding with each of the pollinia which can then grow onto the stigma (Fig. 2c). Most commonly, the flowers higher up the inflorescence lack a rostellum altogether and the pollinia are in close proximity with the stigma allowing for rapid auto-pollination (Fig. 2d). The upper flowers also lack a rostellum, but frequently do not develop pollinia at all (Fig. 2e). These flowers do not set fruit.

Only flowers that lack a rostellum but posses pollinia go on to produce capsules (Fig. 3). These flowers represent the majority (66 to 87%) of flowers produced on the inflorescences of all five taxa examined. Less commonly (11 to 28%) flowers lack a functional rostellum but also do not produce pollinia. None of these flowers produce capsules. In all taxa a small proportion of flowers appear functional with an intact rostellum and one or more pollinia present. None of these flowers in any of the five taxa produced capsules. A few flowers of *E. c. clavicornis* had functional rostellae but did not produce pollinia. None of these flowers subsequently set fruit (Fig. 3a).

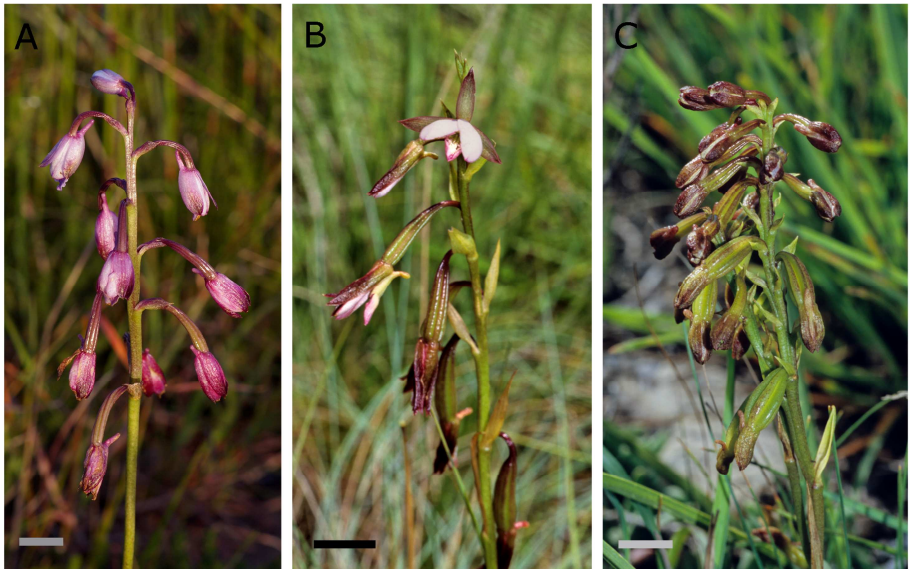

Figure 1: Inflorescences of A) *Eulophia clavicornis* subspecies *clavicornis*, B) *E. clavicornis* subspecies *nutans* and C) *E. tenella* showing very high rates of fruit set indicative of auto-pollination. Bar = 10 mm.

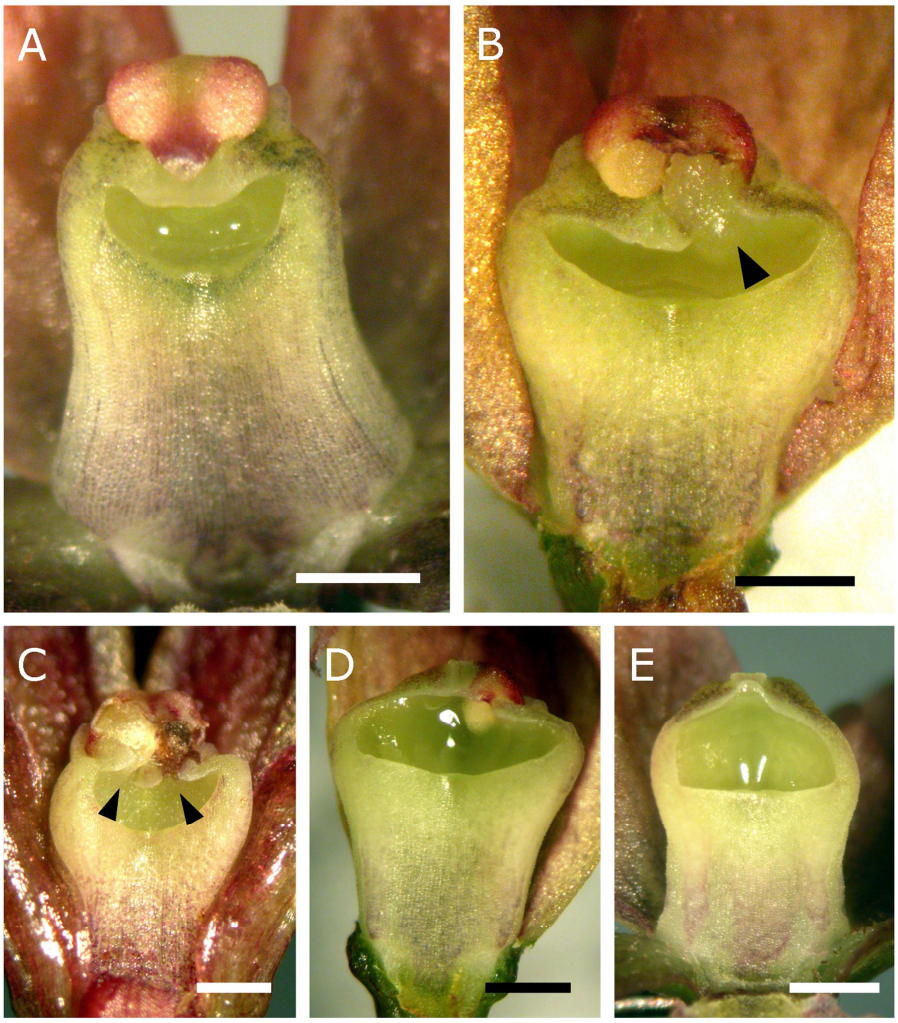

Figure 2: Gynostemium of *E. tenella* showing the range of rostellum reduction from A) with an intact rostellum; B) Rostellum with one gap present (arrow); C) rostellum with two gaps (arrows) allowing pollinia to swell and pollen tubes to grow into the stigma, D) freshly opened flower with rostellum completely absent and the flower producing only one pollinium directly in the stigmatic fluid and E) flower from near the distal end of the inflorescence with no pollinia produced and the rostellum completely absent. Bar = 0.1 mm.

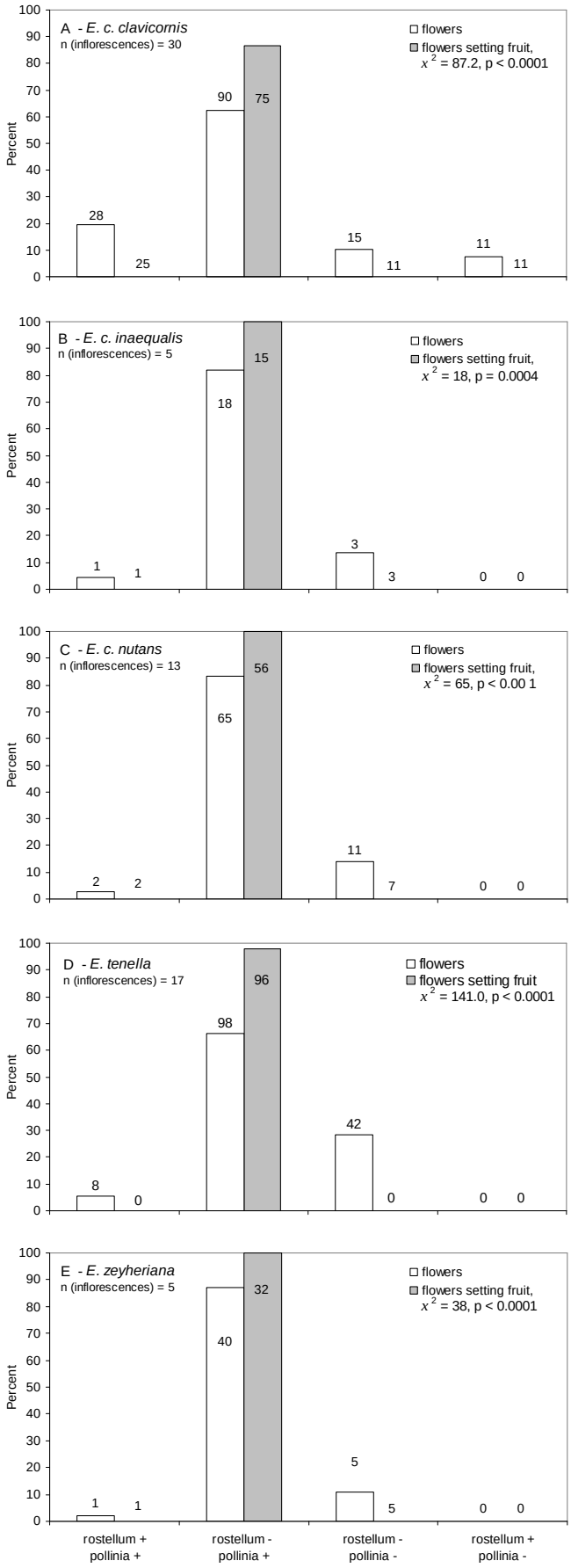

Figure 3: Percentage of flowers with (+) and without (-) pollinia and functional rostellum tissue in the four auto-pollinating taxa as well as the auto-pollinating form of *E. zeyheriana*. Only flowers without a rostellum or with gaps in their rostellum (rostellum -) that produce one or more pollinia (pollinia +; grey bars) go on to produce capsule in these five taxa. Chi-square values compare the frequency of flowers setting fruit versus flowers that did not set fruit across each of the four categories.

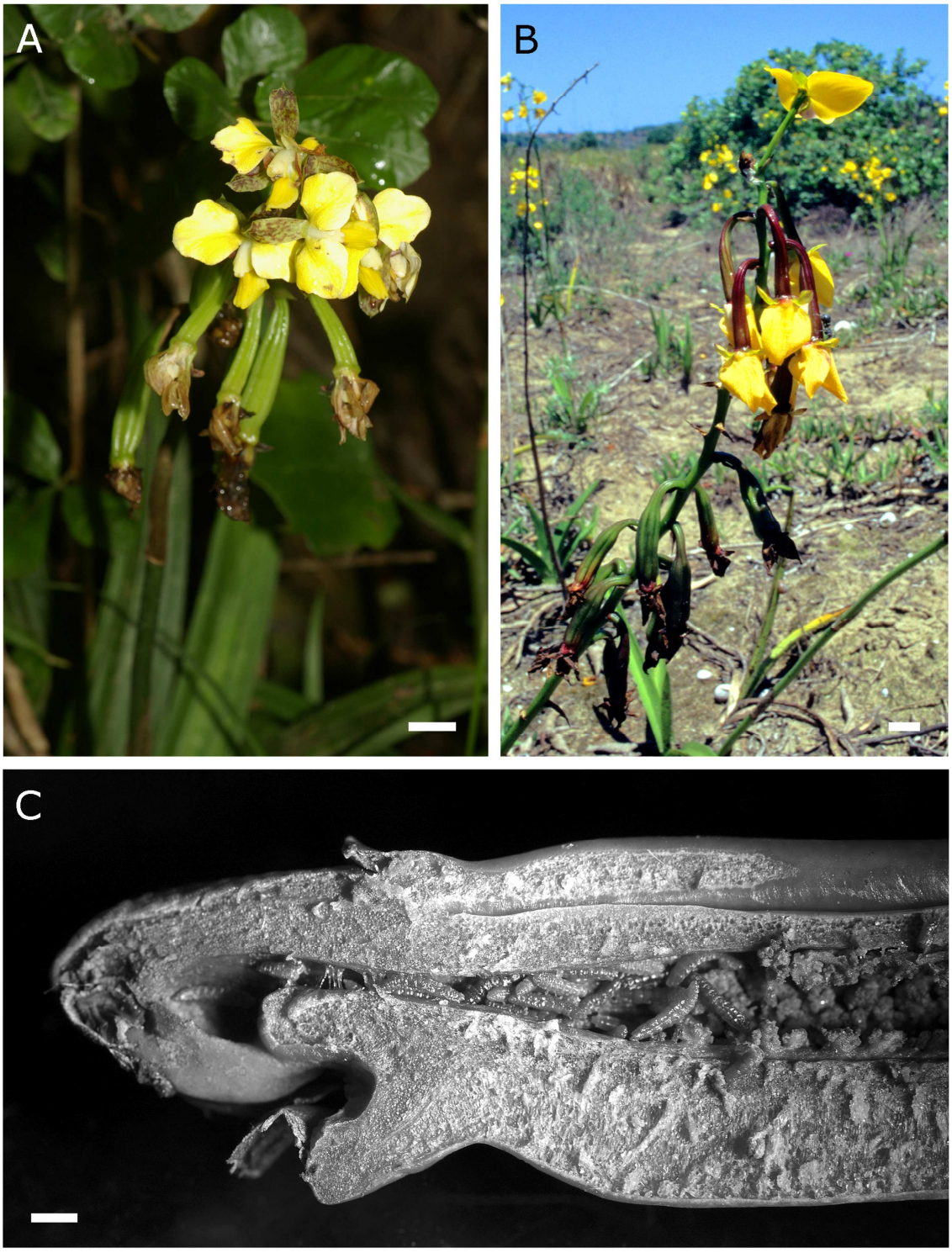

Figure 4: Insect larvae parasitize the ovaries of some species of *Eulophia* causing most flowers on an inflorescence to develop capsules. This has been observed in A) *E. streptopetala* and B) *E. speciosa*. Large numbers of these larvae are found in the ovaries of parasitized flowers such as this *E. speciosa* flower (C). Bar: A & B = 10 mm, C = 1 mm.

Insect parasites cause capsule development

In contrast to these auto-pollinating taxa, high rates of fruit set were recorded in a few individuals of *E. speciosa* (Fig. 4a) and *E. streptopetala* (Fig. 4b) which are xenogamous bee-pollinated species (Chapter 5). This is apparently caused by parasitizing insect larvae that are present in large numbers in the ovaries of the flowers of these plants (Fig. 4c). The flowers concerned show no signs of insect visitation with pollinaria still present and no pollinia deposited on their stigmas. In addition, these species have large flowers with a well-developed rostellum ruling out the mechanism of auto-pollination described above.

Table 2: Results of an experiment to determine the breeding system of *Eulophia clavicornis* var. *nutans*. Means that share superscript letters are not significantly different (ANOVA, followed by Tukey multiple range test).

	Self-pollinated (n = 9)	Cross-pollinated (n = 12)	Bagged and unmanipulated (n = 19)	Emasculated (n = 13)
Capsule Set	100%	100%	100%	0%
Capsule & seed mass (g)	0.130 [a]	0.120[ab]	0.095 [b]	No fruit set
Seed mass (g)	0.029	0.03	0.023	No seed set
% fertile	46.5[ab]	58.1 [a]	40.1 [b]	No seed set

Breeding systems

Controlled pollination experiments involving *E. clavicornis* var. *nutans* indicates that there is no difference in rates of capsule set between bagged self-pollinated, cross-pollinated and unmanipulated flowers of this taxon. However capsule and seed mass was significantly higher in the self-pollinated treatment than in the auto-pollination control. Similarly, the percentage of fertile seeds in cross-pollinated fruits was significantly higher than in auto-pollinated fruits. Our data suggests that agamospermy is unlikely as emasculated flowers failed to set fruit (Table 2).

DISCUSSION

This study, together with that of Williamson (1984) and Catling (1990), increases the number of known auto-pollinating *Eulophia* species to 11, accepting Thomas' (1998) suggestion that two of Williamson's (1984) taxa are synonyms of a third. Auto-pollinating species of *Eulophia* therefore represent 30% of the 37 taxa that have been examined to date (Lock & Profita 1975, Williamson 1984, Catling 1990, as well as the 29 taxa examined during this

study), which is similar to the overall incidence of auto-pollination in the Orchidaceae (Catling 1990, chapter 1).

The mechanism of auto-pollination – reduced rostellae allowing contact between the stigmatic secretions and the pollinia – appears to be the same in the taxa examined here and a number of those described by Williamson (1984). The absence of rostellar tissue allowing contact between stigmatic fluid and pollinia (mechanism 5a of Catling 1990) is by far the commonest mechanism for auto-pollination in the orchids and represents 52% of the 174 species that have had mechanisms assigned by Catling (1990). Some of the taxa described by Williamson (1984) show evidence of a difference mechanism in which the rostellum is intact but stigmatic, stimulating the germination of pollen tubes from the pollinia (mechanism 5b of Catling 1990).

In the absence of a phylogeny for *Eulophia*, it is impossible to precisely determine the number of independent origins of auto-pollination in the genus. The varieties of *Eulophia clavicornis*, *E. tenella* and *E. zeyheriana* are likely to belong to the same clade (although auto-pollination may have had independent origins in this clade). I suspect that some of the species studied by Williamson (1984) are also from this clade, but this remains speculative until a phylogeny becomes available. Auto-pollinating species tend to have a similar appearance (based on their reduced floral displays) and were therefore often misplaced in older phylogenies based on morphological characters (Bytebier *et al.* 2007).

Catling (1990) and Gonzalez & Ackerman (1988) describe auto-pollination in *E. alata* and the related *Oeceoclades maculata* respectively. Auto-pollination in these two species is assured through the action of the stipe that bends to move the pollinia to make contact on the stigma (mechanism 4d of Catling 1990). This is compatible with what is known of the bending mechanism of pollinaria reconfiguration in other *Eulophia* species. The *Eulophia streptopetala*-type reconfiguration described in Chapter 5 would allow pollinia to be repositioned from their undisturbed position at the distal end of the gynostemium, over the rostellar tissue to be in close proximity to the stigmatic cavity and is much the same as the so-called novel mechanism described by Liu *et al.* (2006).

The breeding system conducted for *E. c. nutans* (Table 2) suggests that agamospermy, the other possible explanation for the high rates of capsule set, is unlikely, but I cannot exclude processes such as pseudogamy (Richards 1986). In addition a number of terminal flowers of

each of the five taxa examined lacked pollinia and failed to produce capsules (Fig. 3). These natural emasculation experiments also suggest that agamospermy is unlikely in these taxa. Finally nine flowers of *E. c. clavicornis* showed evidence of natural pollinarium removal from the lower functional flowers and failed to set fruit.

The taxa described here all support a floral "syndrome" of auto-pollination. Key features of such a "syndrome" include: high levels of fruit set; small, dull coloured flowers which often show various deformities to the petals and gynostemium; as well as flowers which hardly open or open only for a brief period. Small flower size is possibly a key preadaptation for the degeneration or absence of the rostellum as a mechanism for auto-pollination. Smaller flowers have correspondingly small rostellae made up of only a few layers of cells which are more likely to be interrupted by developmental anomalies than in large flowers with a substantial rostellum.

Using this "syndrome", albeit without being able to confirm a mechanism, it is possible to predict that another *Eulophia* species, *E. millnei*, is a likely an auto-pollinating species. Photographs of this species in a number of books (La Croix *et al.* 1991, Linder & Kurzweil 1999) show the vast majority of flowers developing capsules. This species has small dull cream-green flowers that open poorly.

High rates of fruit set are obviously one of the key traits of an "auto-pollination syndrome." However, given the action of parasitizing diptera in two xenogamous *Eulophia* species and possibly in *Acrolophia cochlearis* and the absence of viable seed in these "pseudocapsules", this trait needs to be used in conjunction with the determination of mechanisms such as the absence of the rostellum and possibly the behaviour of the pollinarium before inferring auto-pollination in *Eulophia* and related taxa such as species in the genus *Acrolophia*.

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[chapter 9]

Summary and conclusions

In this thesis, I was able to test a number of hypotheses relating to the evolution and ecology of pollination systems, using *Eulophia* as a model system. Here I summarize the support for these hypotheses and highlight the main findings.

I found a limited diversity of pollination systems in *Eulophia*, but, as was hypothesized from floral traits associated with pollination syndromes, bee-pollination systems are the most frequent among the species studied followed by beetle- and auto-pollination. This is in contrast to the orchid genus *Disa* (Johnson *et al.* 1998) and various genera in the Iridaceae (Goldblatt *et al.* 2000, Goldblatt *et al.* 2001, Goldblatt *et al.* 2004, Goldblatt *et al.* 2005, Manning & Goldblatt 2005) which are pollinated by a diverse range of insect orders as well as birds. Over the course of this study, I have been able to document the pollination biology of 15 South African *Eulophia* taxa, thus adding considerably to the initial studies of Lock & Profita (1975) and Williamson (1984). These include detailed accounts of bee-pollination in species including *E. zeyheriana*, a Batesian mimic of *Wahlenbergia cuspidata* pollinated by halictid bees (Peter & Johnson 2008 [chapter 2]); the long-spurred form of *E. parviflora* pollinated by *Amegilla fallax* (Anthophorinae, Apidae; [chapter 6]); and observations of pollination by medium and large xylocopid bees in *E. speciosa*, *E. angolensis* and *E. cucullata*; pollination by large megachilid bees in *E. streptopetala*; halictid bees in *E. o. ovalis* [chapter 7]. This thesis also includes observations of the pollination of *Acrolophia cochlearis* by *Colletes* bees that are rewarded by minute quantities of very concentrate nectar [chapter 5].

Given the apparent rarity of beetle-pollination in the Orchidaceae, the discovery of beetle-pollination in a number of these deceptive species was unexpected. These include *E. foliosa* pollinated by small click beetles (Peter & Johnson 2006a [chapter 3]); the short-spurred form of *E. parviflora* pollinated by *Cyrtothyrea marginalis* beetles (Cetoniinae, Scarabaeidae [chapter 6]); as well as the two colour forms of *E. ensata* and *E. welwitschia* all being pollinated by

cetoniid beetles [chapter 7]. These observations significantly increase the number of known specialist beetle-pollinated orchids.

Besides these insect pollinated species, I also found that the genus includes a number of auto-pollinating taxa. These are *E. tenella* and three varieties of *E. clavicornis* - *E. c. clavicornis*, *E. c. nutans* and *E. c. inaequalis* [chapter 8].

The majority of xenogamous *Eulophia* species appear to employ generalised food deception. This includes the majority of the bee- [chapter 6, chapter 7] and possibly beetle-pollinated species [chapter 3, chapter 6, chapter 7]. In contrast, I found strong support for the hypothesis that the unusually pale blue-coloured flowered species *E. zeyheriana* is a Batesian mimic of the flowers of co-occurring and rewarding *Wahlenbergia cuspidata* (Peter & Johnson 2008 [chapter 2]).

The study of *E. zeyheriana* demonstrates the importance to a mimic of colour-matching and proximity to the rewarding model species. Flower colour appears to be a critical component of pollinator attraction because experimental reduction in ultraviolet reflectance resulted in a significant decrease in visitation to the orchid. This shows the functional importance of colour matching between model and mimic in a floral Batesian mimicry system for the first time. Surveys in natural populations and a translocation experiment showed that proximity to the rewarding models provides uni-directional facilitation of the pollination success of *E. zeyheriana* and significantly increases visitation rates to this species, thus providing support for the magnet species hypothesis, an idea which has been tested experimentally by Johnson *et al.* (2003 [appendix 1a]). Other aspects of the biology of *E. zeyheriana* also support the hypothesis that it is a Batesian mimic of *W. cuspidata*. This includes the fact that the distribution of the orchid as well as its flowering phenology closely matches that of the rewarding model.

Besides the overall colour similarity between model and mimic, colour may also be important in providing cues that give the appearance of a pollen reward (cf. Heuschen *et al.* 2005). Many *Eulophia* species have UV absorbing patches on their labellae that contrast to the colour of the rest of the flower and may represent important traits for generalised deception of pollen collecting pollinators [chapter 7]. A more specific case of pollen mimicry (cf. Nilsson 1983) is the presence of white, UV absorbing patches on the rolled

labellum of the Batesian mimic *E. zeyheriana* that resemble the pollen presenter of the rewarding model *Wahlenbergia cuspidata* (Peter & Johnson 2008 [chapter 2]).

The capitate inflorescences of some beetle-pollinated species such as *E. ensata* are yellow, but strongly UV absorbent unlike other yellow species examined and may be Batesian mimics of rewarding Asteraceae inflorescences [chapter 7] which share such traits and reward their pollinators with abundant, exposed pollen. This requires further investigation.

The vast majority of the *Eulophia* and *Acrolophia* species examined possess pollinarium bending reconfiguration mechanisms, although two different modes of bending reconfiguration have been documented. These include the more common mode named the *Eulophia streptopetala*-type bending mechanism which entails the pollinia being reoriented through approximately 180 degrees from an initial forwards orientation to one pointed backwards relative to the pollinator. In contrast, a few species such as *E. speciosa*, two of its presumed relatives and *Acrolophia cochlearis* have pollinaria that undergo a *Eulophia speciosa*-type reconfiguration: freshly removed pollinaria have their pollinia orientated at right angles to the point of attachment and reconfigure by bending forward through about ninety degree to be correctly orientated to make contact with the large exposed stigmas of these species [chapter 7]. This is analogous to the "depression" movement that Darwin (1867) described for the massulate pollinia of a number of European orchidoid orchids.

A few species, notably *E. foliosa* (Peter & Johnson 2006a, [chapter 3]) and *E. ensata* [chapter 7] have a different primary form of pollinarium reconfiguration – anther cap retention. This mode of pollinarium reconfiguration entails the anther cap clasping onto the pollinia making them too large to be inserted into the stigma. Anther cap retention has only been documented in a few orchid species to date (reviewed by Peter & Johnson 2006a [chapter 3]), although this apparent rarity may be due to the cryptic nature of this mechanism. Peter and Johnson (2006a, [chapter 3]) describe anther cap retention in detail and propose that water loss from specialised tissue of the anther cap is responsible for the delayed dropping of the anther cap tissue in *E. foliosa*.

These observations of pollinarium bending and anther cap retention in a number of *Eulophia* and *Acrolophia* species (described in various chapters of this thesis), along with observations of visit times to the inflorescences of these species by their respective pollinators, allowed us to test Darwin's (1867) hypothesis that these mechanisms protect against self-pollination

(Peter & Johnson 2006b, [chapter 4]). This analysis used not only observations of *Eulophia* and *Acrolophia* species but also a number of other orchids and asclepiads. In all but one of the 19 species pollinarium reconfiguration times exceed the average visit times of pollinators. These data thus strongly support Darwin's (1867) cross-pollination hypothesis for pollinarium reconfiguration and is the only study besides that of Johnson *et al.* (2004, [appendix 1b]) to explicitly test Darwin's idea.

While all of the 25 species of *Eulophia* examined are deceptive, two of the three examined species in the small, closely related Cape genus *Acrolophia* provide their pollinators with a small highly concentrated nectar reward [chapter 5]. The rewarding species, *A. cochlearis*, exhibits very high rates of pollen transfer efficiency relative to those recorded in the deceptive species *A. capensis* and the deceptive species of *Eulophia*. This difference is probably because nectar rewards encourage foraging constancy. However, rewarding species may also be expected to have higher rates of geitonogamous self-pollination due to prolonged foraging. A high proportion of naturally pollinated fruit appear to result from self-pollination in one population of *A. cochlearis*, providing some support for this idea. Rates of self-pollination were determined using a novel method which utilises the differential rates of embryo abortion in experimentally cross- and self-pollinated flowers as a signal to calibrate rates of self-pollination in naturally pollinated fruits. This is a potentially powerful approach that may prove very useful for determining rates of self- and cross-pollination in epidendroid orchids, a large and important group in the family.

In chapter 6 the evolutionary divergence of long- and short-spurred forms of *E. parviflora* in response to different pollinators is investigated. Each of these two forms represent a pollination ecotype, with clear evidence that divergence has occurred in floral morphology, scent chemistry and flowering phenology and that this divergence can be attributed to adaptations to the respective bee and beetle pollinators of each form. Thus, the initial hypothesis of a link between floral divergence and a pollinator shift in *E. parviflora* was strongly supported. Choice experiments in a y-maze olfactometer showed that beetles are preferentially attracted to the scent of the short-spurred form. A spur-shortening experiment showed that long spurs are required for effective pollination of the bee-pollinated form. The two forms have different distribution and it was hypothesised that this reflects differing distributions of the respective pollinators. However, a transplant experiment did not support this idea. An alternative explanation is that early flowering in the long-spurred form is an adaptation to exploit the emergence of naïve bees which in turn restrict this form to

coastal areas where frost is absent. Later flowering of the short-spurred form coincides closely with the emergence of the pollinating beetles following winter frosts.

This study on the pollination biology of a number of *Eulophia* species has not only shed light on the pollination of a genus poorly-studied in terms of reproductive biology, but has also advanced theoretical ideas about the evolution of floral deception, rates of cross-pollination and role of pollinators in plant diversification. The priority now is to find gene regions with sufficient sequence variation to allow the construction of a robust phylogeny of *Eulophia*. A phylogeny will be invaluable for deciphering the overall role of pollinators in driving the evolution of the substantial floral diversity in this genus.

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Appendix 1: related papers

[appendix 1a]

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POLLINATION SUCCESS IN A DECEPTIVE ORCHID IS ENHANCED BY CO-OCCURRING REWARDING MAGNET PLANTS

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Abstract. It has been debated whether pollination success in nonrewarding plants that flower in association with nectar-producing plants will be diminished by competition for pollinator visits or, alternatively, enhanced through increased local abundance of pollinators (the magnet species effect). We experimentally evaluated these effects using the nonrewarding bumblebee-pollinated orchid *Anacamptis morio* and associated nectar-producing plants at a site in Sweden. Pollination success (estimated as pollen receipt and pollen removal) in *A. morio* was significantly greater for individuals translocated to patches of nectar-producing plants (*Geum rivale* and *Allium schoenoprasum*) than for individuals placed outside (~20 m away) such patches. These results provide support for the existence of a facilitative magnet species effect in the interaction between certain nectar plants and *A. morio*. To determine the spatial scale of these interactions, we correlated the visitation rate to flowers of *A. morio* with the density of sympatric nectar plants in 1-m^2 and 100-m^2 plots centered around groups of translocated plants, and at the level of whole meadows (~0.5–2 ha). Visitation rate to flowers of *A. morio* was not correlated with the 1-m^2 patch density of *G. rivale* and *A. schoenoprasum*, but showed a significant positive relationship with density of these nectar plants in 100-m^2 plots. In addition, visitation to flowers of *A. morio* was strongly and positively related to the density of *A. schoenoprasum* at the level of the meadow. Choice experiments showed that bees foraging on the purple flowers of *A. schoenoprasum* (a particularly effective magnet species) visit the purple flowers of *A. morio* more readily (47.6% of choices) than bees foraging on the yellow flowers of *Lotus corniculatus* (17% of choices). Overall similarity in flower color and shape may increase the probability that a pollinator will temporarily shift from a nectar-producing "magnet" plant to a nonrewarding plant. We discuss the possibility of a mimicry continuum between those orchids that exploit instinctive food-seeking behavior of pollinators and those that show an adaptive resemblance to nectar-producing plants.

Key words: Anacamptis morio; Bombus; *competition; magnet species; mimicry; nectar; Orchidaceae; pollen limitation; population density; population size; transplant experiment.*

INTRODUCTION

Interactions between plants and animals may be strongly influenced by the local physical and biotic environment (Thomson 1978, Campbell 1987, Johnson and Bond 1992, O'Connell and Johnston 1998). Proximity to other plant species, in particular, has been shown to affect the intensity of pollination or herbivory experienced by a focal species (Thomson 1978, Root 1973, Holt and Lawton 1994, Callaway 1995, Hambäck et al. 2000). Pollination success may be diminished by competition, either when neighboring plants with superior rewards draw pollinators away, or when sharing of pollinators results in reproductive interference through the receipt of heterospecific pollen, or wasted export of pollen to heterospecific stigmas (Free 1968, Waser 1983). Many plant traits, including flowering time, floral specialization for specific pollinators, and even habitat requirements, have been interpreted as evolutionary outcomes of competition among plants for pollinators (Heinrich 1975).

Although interactions between coflowering plants have tended to be viewed within the paradigm of competition, there is increasing recognition that some plants may actually facilitate the pollination of others in the same community (Feinsinger et al. 1986, Feinsinger 1987). One mechanism for facilitation is the "magnet species effect" (Thomson 1978) whereby a rewarding species increases the pollination success of neighboring plants with inferior rewards. The magnet species, as its name implies, may function by increasing the local abundance of pollinators. Neighboring plants may gain a net benefit from the greater abundance of pollinators around the magnet, even though pollinators may show more or less constancy to the magnet species and cause reproductive interference. Nonrewarding plant species may benefit most from close proximity to magnet species, although the phenomenon may extend to plants with floral rewards (Pellmyr 1986, Laverty 1992).

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Plants that do not produce floral rewards are surprisingly common, and include approximately one-third of all orchids, by some estimates the largest angiosperm family (Dafni 1984, Ackerman 1986). Some of these deceptive plants are spectacular mimics of co-occurring food plants or even female insects, but the large majority simply exploit the instinctive food-seeking behavior of pollinators, a phenomenon termed generalized food deception (Nilsson 1992). It seems reasonable that deceptive plants would compete poorly with nectar plants for pollinator visits. The common occurrence of deceptive orchids in habitats lacking many rewarding plants, such as marshes, has been considered as consistent with the competition hypothesis (Heinrich 1975, Nilsson 1980, Firmage and Cole 1988). The competition hypothesis has received empirical support from decreased pollination success in patches of the deceptive marsh orchid *Dactylorhiza incarnata* following experimental addition of nectar-producing *Viola* flowers (Lammi and Kuitunen 1995). Lammi and Kuitunen (1995) referred to increased success of their orchids in the absence of rewarding plants as the "remote habitat" effect. On the other hand, Laverty's (1992) study, showing that pollination success in non-rewarding mayapples *Podophyllum peltatum* L. is positively related to the proximity of a cluster of nectar-producing lousewort *Pedicularis canadensis* L. colonies, supports the alternative facilitation hypothesis and, more specifically, the magnet species effect proposed by Thomson (1978). In another test of the magnet species effect, Alexandersson and Ågren (1996) found that pollen export from flowers of the deceptive orchid *Calypso bulbosa* L. was positively related to the density of the nectar-producing plant *Salix caprea* L. in one of three years of study.

In light of the equivocal evidence for these two contrasting hypotheses (competition vs. facilitation), it has not been possible to generalize about the importance of rewarding plants for the pollination success of sympatric nonrewarding plants. Furthermore, we know little about the spatial scale and density dependence of such interactions. Do food-deceptive species depend on rewarding species in the larger habitat (on the scale of hectares), yet benefit from growing in smaller habitat units, such as marshes, that have few rewarding species, or do food-deceptive species gain benefit from intermingling with rewarding species at all spatial scales and densities? Finally, we know even less about the role of floral traits in determining the outcomes of these interactions. For example, does similarity in color and shape between a deceptive and rewarding species increase the likelihood that the rewarding species will act as a magnet species?

The aims of this study were (1) to test whether the pollination success of a nonrewarding orchid is enhanced or diminished by the presence of nectar producing plants, (2) to determine the influence of both spatial scale and plant density on these interactions, and (3) to gain some insights into whether floral traits (and their influence on pollinator behavior) could explain why some plants might act as magnet species while others do not.

Methods

The study species

Fieldwork took place during May through June 2001 on the island of Öland off the east coast of Sweden. The study species, *Anacamptis morio* (L.) Bateman, Pridgeon & M. W. Chase (syn. *Orchis morio* L.), occurs in large populations in virtually all of the open grazed meadows in the vicinity of the Ecological Field Station of Uppsala University at Ölands Skogsby. This orchid is nonrewarding and pollinated almost exclusively by queen bumblebees at this site (Nilsson 1984). As the purple-pink flowers of *A. morio* do not closely resemble the color or shape of flowers of any sympatric rewarding species, its pollination system has been characterized as generalized food deception (Nilsson 1984). Queen bumblebees in the meadows around the Ecological Research Station feed mainly on nectar in the flowers of *Geum rivale* L. (Rosaceae), *Anthyllis vulneraria* L. (Fabaceae), *Lotus corniculatus* L. (Fabaceae), and *Allium schoenosprasum* L. (Alliaceae) during the time that *A. morio* is in flower (Fig. 1, Nilsson 1984). *Anacamptis morio* grows intermingled to a greater or lesser degree with all of these species, except *G. rivale*, which tends to occupy deeper soils than the orchid.

Previous studies have shown that *A. morio* is self-compatible, but relies on pollinator visits for fruit set (Nilsson 1984). Like many deceptive orchids, fruit set in *A. morio* is strongly pollen limited, and ~50% of the plants fail to produce fruits in a given year (Nilsson 1984, Johnson and Nilsson 1999).

Translocation experiments

We carried out two separate translocation experiments to determine the consequences for pollination success of individual plants of *A. morio* when they flower in patches of bumblebee-pollinated nectar plants vs. when they flower outside such patches.

In the first experiment, involving *G. rivale* as the nectar plant, 168 orchids were excavated in sods of original turf and potted individually at the beginning of the flowering season. All flowers on the experimental plants were checked, and those with pollen on the stigma or pollinia removed were excised. The plants were then randomly assigned to one of two treatments in a paired design with each replicate consisting of four plants, placed 50 cm apart in a square configuration, in a flowering patch of *G. rivale* and another four plants placed in the same configuration ~20 m away from the patch. This "remote" location matched the patch location in terms of physical and vegetation attributes, but lacked flowers attractive to bumblebees. Pots were

FIG. 1. *Bombus lapidarius* queens visiting flowers of the plant species used in this study: (A) *Anacamptis morio*; (B) *Lotus corniculatus*; (C) *Geum rivale*; (D) *Anthyllis vulneraria*; (E) *Allium schoenoprasum*. Scale bars are each 10 mm.

buried in the soil and the orchids did not require watering throughout the 10-day duration of the experiment, which was characterized by intermittent rainfall. Twenty-one pairs were placed in the meadows around the field station, each using a separate patch of *G. rivale*. The mean number of flowers per plant did not differ significantly between the treatments (9.4 inside vs. 8.8 outside patches of *G. rivale*; paired *t* test, $t = 1.1$, df = 19, $P = 0.27$). The orchids were replanted in their original habitat once flowering was completed.

In the second experiment, involving *A. schoenoprasum* as the nectar plant, 112 orchid inflorescences were cut at ground level and placed in film canisters filled with florist's foam and water. Flowers with pollen on the stigma or pollinia removed were excised. Each inflorescence was then randomly assigned to one of two treatments in a paired design consisting of two inflorescences placed 50 cm apart in a flowering patch of *A. schoenoprasum*, and two inflorescences placed in the same configuration, but ~20 m away in a location that was matched in terms of physical and vegetation attributes, but lacked any flowering plants attractive to bumblebees. The film canisters were buried at ground level and topped up with water daily. Twenty-eight pairs, each utilizing a separate patch of *A. schoenoprasum*, were placed in the meadows around the field station. Flower number per inflorescence did not differ significantly between the two treatments (7.6 inside vs. 7.5 outside patches of *A. schoenoprasum*; paired *t* test, $t = 0.23$, df = 26, $P = 0.82$). The inflorescences were harvested after three days when they started to show signs of wilting.

At the end of both translocation experiments, we recorded for each inflorescence the number of flowers with conspecific pollen on the stigma, the number of flowers with pollinia removed, and the total number of flowers with signs of visitation (either pollen deposited, pollinia removed, or both). As groups were treated as replicates, we calculated means of each measure from the individual plants in a group. We used paired *t* tests to examine whether the proportion of stigmas pollinated, proportion of flowers from which pollinia were removed, and mean overall visitation (proportion of flowers showing signs of either pollination or pollinia

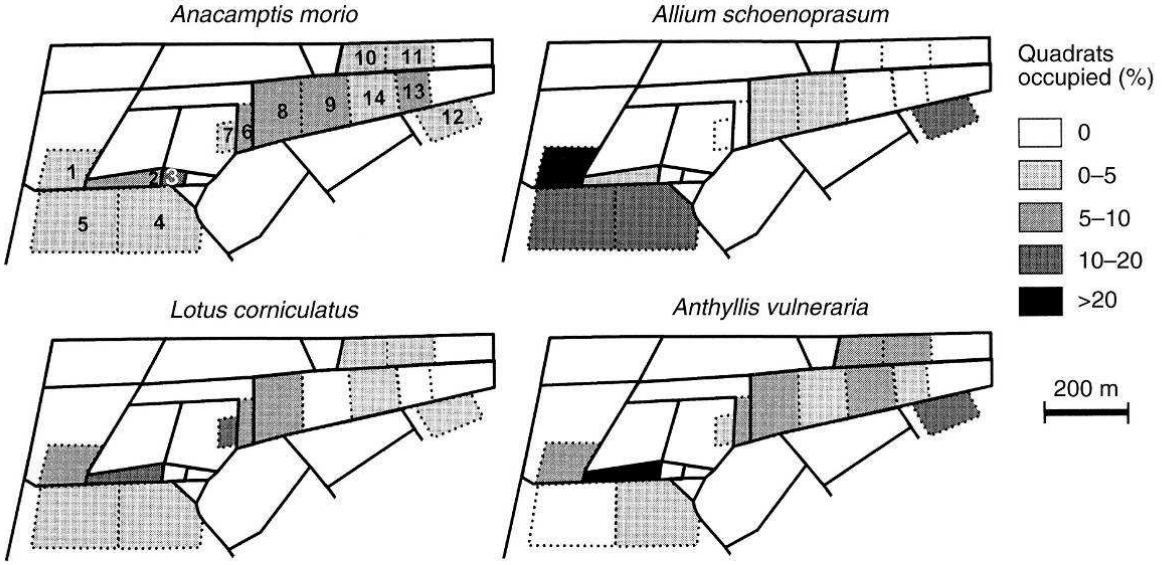

FIG. 2. Distribution and relative density (percentage of occupied 0.4-m^2 quadrats) of the study species in 14 meadows (numbered) at the study site. Thick black lines indicate meadows bounded by stone walls and hedgerows.

removal) differed between groups of translocated orchids placed inside and outside patches of nectar plants. We also recorded the density of nectar plants (flowering stems) in square plots of 1 and 100 m^2 around each group of orchids translocated into patches of nectar plants. The effect of nectar plant density on the proportion of flowers visited, proportion of flowers receiving pollen, and proportion of flowers from which pollen was removed was analyzed using linear regression (analyses based on means for each group of translocated orchids). All proportions were arcsine-square-root transformed prior to analysis.

Meadow surveys

To determine whether the pollination success of *A. morio* is influenced by the nectar plant density at a larger habitat scale than examined in the translocation experiments, we carried out a survey of the density of *A. schoenoprasum*, *A. vulneraria*, and *L. corniculatus* in 14 meadows in which *A. morio* occurs close to the Ecological Field Station (Fig. 2). Meadows at this site are surrounded by stone walls overgrown with shrubs and trees and can differ markedly in the abundance of nectar plants as a result of differing management practices. Three exceptionally large or narrow meadows were divided a priori into several smaller meadows that shared a common boundary without a stone wall (Fig. 2). We laid out parallel transects across each meadow at 10-m intervals and recorded the occurrence of the three nectar plants as well as *A. morio* in 0.4-m^2 quadrats spaced at 1-m intervals along each transect. Orchids encountered along these transects were scored for pollination success in the same manner as those in the translocation experiments. If few orchids were encountered along these transects, additional transects were laid out until the total number of orchids scored per meadow reached at least 20.

We used multiple regression to examine how measures of pollination success (proportion of flowers visited, proportion of flowers receiving pollen, and proportion of flowers from which pollen was removed) were related to five meadow variables (densities of the three nectar plants, density of orchids, and meadow size). Univariate regression was used to explore the relationship between overall nectar plant density and the proportion of orchid flowers visited. All proportions were arcsine-square-root transformed prior to analysis.

Choice experiments

Pollinators of deceptive orchids are very seldom observed visiting orchid flowers. For example, Nilsson (1984) recorded sequences of bumblebee visits to flowers of *A. morio* on only eight occasions during 10 years of observations on Öland. To establish the frequency with which queen bumblebees actually accept or reject orchid inflorescences encountered along their foraging routes, we used the "bee interview technique" (Thomson 1988, Johnson and Nilsson 1999). A freshly cut inflorescence with 10 flowers (the average for this population) was tied to the end of a long bamboo rod and placed along the foraging path of individual *Bombus lapidarius* queens (the primary pollinator of *A. morio* at this site, according to Nilsson [1984]). The inflorescence was placed 20 cm away from a bee as it foraged on a flower of a nectar plant, and in such a manner that the bee had a choice to visit the orchid or skip it completely and visit another flower of the nectar plant. We also recorded the number of orchid pollinia on the

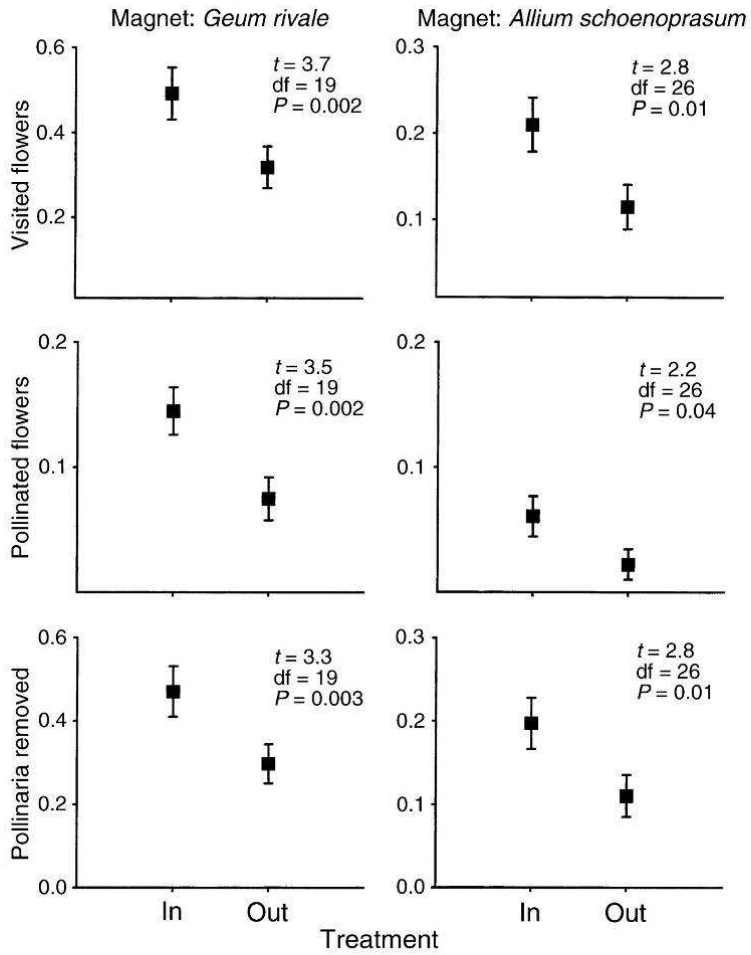

FIG. 3. Mean pollination success (proportion of flowers visited, proportion of flowers pollinated, and proportion of flowers with pollinia removed) of the deceptive orchid *Anacamptis morio* translocated inside (In) or outside (Out) patches of nectar-producing plants. Differences in mean pollination success were examined with paired *t* tests of arcsine-square-root transformed data (*G. rivale*, $n = 21$ groups of translocated orchids; *A. schoenoprasum*, $n = 28$ groups).

bees that made these choices. The choice experiments were all conducted over one 48-h period (5 and 6 June) in one meadow to allow comparisons of the behavior of bees feeding on various nectar-producing plants without introducing seasonal changes in bee behavior as a confounding factor. Chi-square tests were used to examine whether the likelihood that a bumblebee would visit *A. morio* was influenced by the nectar plant on which it was feeding (*A. schoenoprasum* or *L. corniculatus*), or by whether it carried pollinia or not.

RESULTS

Translocation experiments

Orchids translocated into patches of nectar plants performed significantly better in terms of pollen receipt, pollen removal, and overall visitation than those translocated outside such patches (Fig. 3).

For plants translocated to patches of nectar-producing plants, the proportion of flowers visited was not related to the density of nectar plants in the 1-m^2 plots for patches of either *Geum rivale* (linear regression, $R^2 = 0.13$, $P = 0.11$) or *Allium schoenoprasum* ($R^2 = 0.001$, $P = 0.88$). However, the visitation rate to orchid flowers was positively related to nectar plant density in the 100-m^2 plots for patches of both *G. rivale* and *A. schoenoprasum* (Fig. 4).

Meadow surveys

Multiple regression showed that a high percentage of the variation in pollination success of *A. morio* among meadows at the study site can be explained by a model that includes density of the three nectar plants, orchid density, and meadow size (Table 1). Density of *A. schoenoprasum* consistently had a strong positive effect on all measures of pollination success of the orchid (Table 1), while density of *Anthyllis vulneraria* had a significant positive effect on orchid visitation, but no statistically significant effect on the individual

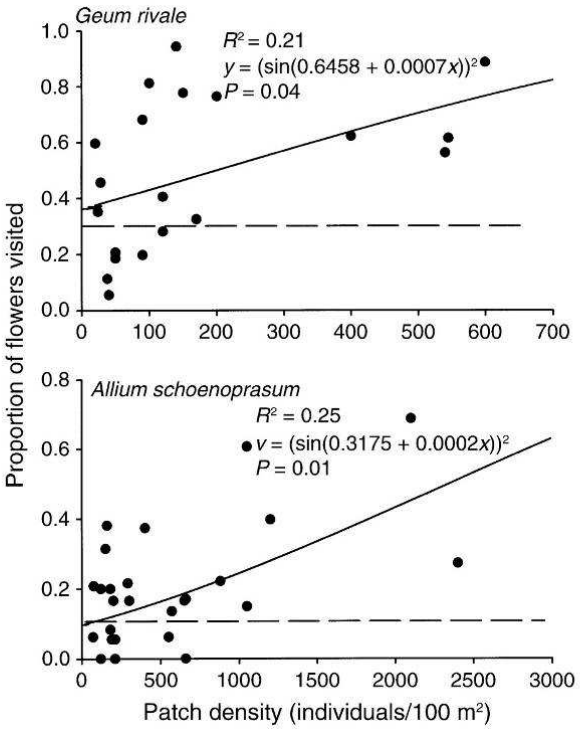

FIG. 4. The relationship between density of nectar plants in 100-m² quadrats and the mean proportion of flowers visited on translocated plants of the deceptive orchid *Anacamptis morio*. The dashed line represents the mean proportion of flowers visited on plants translocated into adjacent habitats lacking nectar plants (Fig. 3).

components of pollination success (pollen receipt and removal). Density of *Lotus corniculatus* had a significant negative effect on pollen deposition, but no significant effect on other measures of pollination success. Orchid density and meadow size did not have significant effects on pollination success in the orchids. Overall, much of the variation in the proportion of *A. morio* flowers visited could be explained by a simple univariate linear relationship with bumblebee nectar plant density in the meadows (Fig. 5).

Choice experiments

We found that *Bombus lapidarius* queens readily visited inflorescences of *A. morio* that were placed along

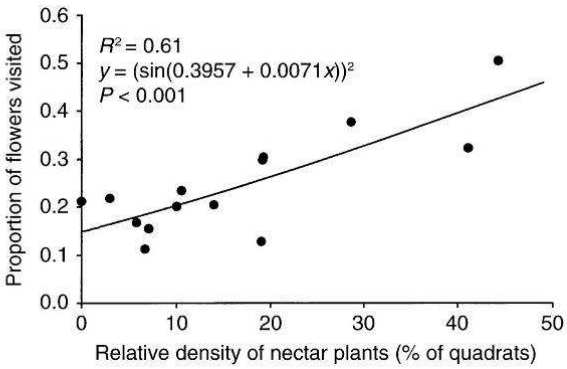

FIG. 5. The relationship between relative density (percentage of occupied 0.4-m² quadrats) of nectar-producing plants and mean proportion of flowers visited on inflorescences of *Anacamptis morio* in 14 meadows at the study site (see Fig. 2).

their foraging routes (Fig. 6). In total, we presented bees with 105 choices of which 35.2% resulted in a visit to the orchid. Bees foraging on the flowers of *A. schoenoprasum* were more likely to visit the orchid than bees foraging on *L. corniculatus* (47.6% vs. 17%). Bees carrying pollinia (indicating that they had previously visited *A. morio*) were more likely to visit the orchid than those without pollinia (Fig. 6).

DISCUSSION

The results of this study are consistent with the magnet species effect proposed by Thomson (1978). Pollination success in *Anacamptis morio* was significantly enhanced by translocation into patches of nectar plants, and positively correlated with density of nectar plants at both the local habitat (100 m²) and the meadow scales. Thus we are able to reject the competition hypothesis and, more specifically, the "remote habitat" effect (Lammi and Kuitunen 1995) as an explanation for spatial patterns of pollination success in this orchid species.

It is of interest to consider why, in light of these results, many, if not the majority, of biologists working on orchid pollination have held the belief that generalized food deceptive orchids face competition from sympatric nectar-producing plants, and thus benefit from growing in "remote" habitats away from such

TABLE 1. Multiple regression models for factors influencing three measures of pollination success (proportion of flowers visited, proportion of flowers pollinated, and proportion of flowers with pollinia removed) in *Anacamptis morio* (analyses based on mean pollination success in 14 meadows).

	Partial regression coefficients					Model		
Dependent variable	ALL	ANT	LOT	ANA	SIZ	F	P	R^2
Visitation	1.18***	0.70*	−0.31	0.68	-0.90×10^{-5}	11.80	0.002	0.81
Pollination	1.00***	0.36	−0.92*	0.42	-0.30×10^{-4}	9.19	0.004	0.76
Pollinia removal	1.08***	0.63	−0.18	0.66	-0.83×10^{-5}	7.86	0.006	0.73

Notes: Degrees of freedom for all three F values are 5 and 8. Abbreviations are: ALL, density of *Allium schoenoprasum*; ANT, density of *Anthyllis vulneraria*; LOT, density of *Lotus corniculatus*; ANA, density of *A. morio*; SIZ, meadow size.
* $P < 0.05$; *** $P < 0.001$.

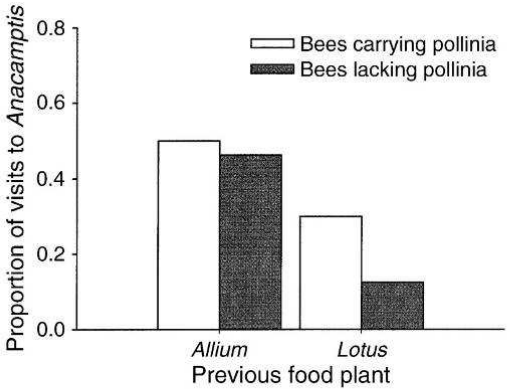

FIG. 6. Results of choice experiments with *Bombus lapidarius* queens using the bee interview technique. Bees foraging on flowers of *Allium schoenoprasum* were more likely to visit *Anacamptis morio* than bees foraging on *Lotus corniculatus* ($\chi^2 = 12.5$, df = 1, $P = 0.008$). Overall, bees carrying pollinia were more likely than bees without pollinia to visit *A. morio* ($\chi^2 = 7.33$, df = 1, $P = 0.006$).

nectar plants (Heinrich 1975, Boyden 1980, Nilsson 1980, Dafni 1984, Firmage and Cole 1988, Lammi and Kuitunen 1995). The origins of the remote habitats hypothesis can be traced to Delpino (1874) who argued that the common pattern in deceptive orchids for fruits to be set only on the lowermost flowers of inflorescences reflects visitation happening only early in the season when insects are inexperienced and not yet drawn away by nectar-producing plants. During the late 1970s and early 1980s, many plant reproductive ecologists were engaged in studying whether staggered flowering patterns are a response to competition for pollinators (Heinrich 1975, Rathke 1988). Flowering of food-deceptive orchids in early spring was interpreted as an adaptation that reduced competition with nectar-producing plants for pollinator visits and maximized the exposure of these plants to visits by inexperienced newly emerged insects (cf. Nilsson 1980). However, more recent studies have recognized that flowering patterns may reflect factors such as phylogenetic constraints and optimal timing for seed dispersal, as much as competition for pollinators (cf. Johnson 1993). Furthermore, the notion that pollinators of generalized food-deceptive orchids are inexperienced is not supported by the present study. The *Bombus lapidarius* queens that visited *A. morio* in early June had probably emerged for several weeks, and yet investigated and probed flowers of *A. morio* when foraging in dense stands of nectar-producing plants (Fig. 6). Most striking was the fact that queens carrying pollinia (and thus those that had clearly visited the orchid previously) were more likely to visit the orchids than those that did not carry pollinia (Fig. 6). While queen bumblebees may learn to avoid orchid flowers in dense monospecific populations (Smithson and Macnair 1997, Ferdy et al. 1998), it seems that orchids intermingled at low densities with nectar-producing plants may continue to benefit from exploratory visits by bumblebees.

In contrast to the results of the present study, Lammi and Kuitunen (1995) found that the pollination success of the deceptive orchid *Dactylhoriza incarnata* was depressed by addition of potted *Viola* flowers. In the latter study, the densities of orchids and nectar plants were low and roughly equal (0.4–0.5 plants/m^2). By contrast, the density of nectar plants far exceeded that of the orchids in the present study, which perhaps better approximates the natural situation for most orchids. It is doubtful whether the potted violas in the study by Lammi and Kuitunen (1995) represented a magnet resource capable of creating a local abundance of bumblebees. Rather, the violas may have acted as a distraction from the orchids for bumblebees that were in transit across the habitat. We found that the intercept for the curve for the relationship between nectar plant density and pollination success of translocated orchids closely matched the basal level of pollination success attained for groups of translocated plants in remote habitats (Fig. 4). This suggests that substantial numbers of nectar plants need to be added to orchid patches if a magnet species effect is to be detected.

Thomson (1981) first considered the spatial scale at which flower-feeding insects might assess the profitability of rewarding patches. He concluded that bumblebees responded to the density of flowering daisy plants in blocks that were ~500 m^2 in size. Our results show that the pollination success of *A. morio* is positively correlated with magnet plant density at both the local habitat (100 m^2) and larger habitat (meadows ~1 ha in size) scales, but uncorrelated with magnet plant density at the microhabitat (1 m^2) scale. A similar lack of correlation between orchid pollination success and food plant density at the 1 m^2 scale was recently reported by Gumbert and Kunze (2001) for the food deceptive orchid *Orchis boryi*, but nectar plant density at larger spatial scales was not recorded in their study. The correlations we observed between nectar plant density and orchid pollination success probably reflect aggregation of queen bumblebees in rewarding patches. The scale of the entire study area, including the 14 meadows, was within the foraging range of individual bumblebees (Osborne et al. 1999), and thus the patterns of pollination success in the orchids were unlikely to be influenced by geographical differences in the abundance of resident bumblebees. The underlying basis of the magnet species effect is almost certainly that some pollinators show greater constancy to individual rewarding sites than they do to individual plant species (cf. Thomson 1981). Recent studies have shown that *Bombus lapidarius* workers show striking constancy to patches containing flowering plants, even when these patches are contiguous (Osborne and Williams 2001).

Our study provides strong indications that not all nectar-producing species will act as magnet species, in

the sense of imparting pollination benefits to neighboring plants with inferior rewards. In the multiple regression of the meadow data, only *A. schoenoprasum* emerged as having consistently strong beneficial effects on the pollination success of *A. morio*. Is it possible to identify traits that make one species more likely to act as a magnet species than another?

Bombus lapidarius queens foraging on the pink–purple flowers of *A. schoenoprasum* (which emerged as a strong magnet species in this study) were more than twice as likely to visit flowers of *A. morio* as were queens foraging on the yellow flowers of the legume *L. corniculatus* (Fig. 6). The flowers of *A. schoenoprasum* are much closer in color to the flowers of *A. morio* in the bee visual spectrum than are the flowers of *L. corniculatus* (Nilsson 1984). We can only speculate as to the role of color in these bee choices because of additional differences in plant height, flower shape, and odor between the species. However, the results are consistent with those of Wilson and Stine (1996) and Chittka et al. (1997), who both showed that bumblebees are more likely to shift to flowers that are similar in color to those on which they have recently been foraging (see also Gumbert 2000). Thus food deceptive orchids are probably more likely to benefit from association with a nectar-producing species that is similar in color (Gumbert and Kunze 2001). This raises the intriguing possibility of a continuum between generalized food deception and true adaptive resemblance. Selection may favor increasing similarity of deceptive orchids to the most effective magnet species with which it shares a common habitat. This might provide a pathway along which evolution may proceed to the well-developed examples of species-specific Batesian floral mimicry (cf. Johnson 1994, Roy and Widmer 1999). If, on the other hand, orchids do not coexist with a stable assemblage of species, then selection would favor a generalized set of display traits that allows the orchid to exploit a number of possible magnet species. The latter scenario seems more likely in the European flora, which is characterized by floral assemblages which are unlikely to have remained stable in the face of postglacial and anthropogenic changes.

An explicit prediction of any mimicry hypothesis is that mimics should enjoy greater fitness when occurring with their model than when alone (Dafni 1984). Thus, increased pollination success in deceptive orchids when growing together with certain nectar plants has been used to argue for the existence of specific Batesian mimicry in flowers (cf. Dafni and Ivri 1981, Dafni 1983, Nilsson 1983, Johnson 1994). Our results suggest that this pattern may be a general phenomenon that occurs even when orchids do not closely resemble nectar flowers. This does not mean that true adaptive resemblance does not occur, but rather that additional evidence must be sought before it is concluded that orchids bear an adaptive resemblance to a particular plant species. Such evidence might include very close matching of spectral reflectance (cf. Nilsson 1983, Johnson 1994), behavioral experiments that establish that pollinators are literally unable to distinguish mimic from model (Johnson 1994, 2000), or a correlation between among-population variation in attractive traits in the putative mimic and variation in the attractive traits of model species (Johnson 1994). Such criteria have seldom been met in any studies of floral mimicry. Indeed, some published studies of floral mimicry may need to be reinterpreted as being a magnet species effect between an orchid and a nectar plant with which it shares a nonspecific overall resemblance.

As was first pointed out by Thomson (1982), we have no reliable way of predicting whether the interaction of any two plant species will be characterized by competition or facilitation for pollination. Orchids with their pollen aggregated into pollinia may be relatively buffered from reproductive interference, as pollen wastage to foreign stigmas generally does not take place (other orchids excepted), and their broad stigmas seldom become clogged with foreign pollen (Harder and Thomson 1989, Johnson and Edwards 2000). In this sense, orchids may benefit more than other plant families from the magnet species effect. However, benefits from magnet species may extend to both nonrewarding (Pellmyr 1986, Laverty 1992) and rewarding (Thomson 1978) plants in other families, and even include fungi with pseudoflowers (Roy 1994). Because of the deeply entrenched notion that plant interactions for visits by pollinators are characterized by competition, ecologists may often have overlooked, or omitted to test for, facilitation in plant assemblages that share common pollinators.

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[appendix 1b]

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The effects of nectar addition on pollen removal and geitonogamy in the non-rewarding orchid *Anacamptis morio*

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It has been suggested that the absence of floral rewards in many orchid species causes pollinators to probe fewer flowers on a plant, and thus reduces geitonogamy, i.e. self-pollination between flowers, which may result in inbreeding depression and reduced pollen export. We examined the effects of nectar addition on pollinator visitation and pollen transfer by tracking the fate of colour-labelled pollen in *Anacamptis morio*, a non-rewarding orchid species pollinated primarily by queen bumble-bees. Addition of nectar to spurs of *A. morio* significantly increased the number of flowers probed by bumble-bees, the time spent on an inflorescence, pollinarium removal and the proportion of removed pollen involved in self-pollination through geitonogamy, but did not affect pollen carryover (the fraction of a pollinarium carried over from one flower to the next). Only visits that exceeded 18 s resulted in geitonogamy, as this is the time taken for removed pollinaria to bend into a position to strike the stigma. A mutation for nectar production in *A. morio* would result in an initial 3.8-fold increase in pollinarium removal per visit, but also increase geitonogamous self-pollination from less than 10% of pollen depositions to *ca.* 40%. Greater efficiency of pollen export will favour deceptive plants when pollinators are relatively common and most pollinaria are removed from flowers or when inbreeding depression is severe. These findings provide empirical support both for Darwin's contention that pollinarium bending is an anti-selfing mechanism in orchids and for the idea that floral deception serves to maximize the efficiency of pollen export.

Keywords: deception; Orchidaceae; pollen carryover; pollination; pollinia; pollinator behaviour

1. INTRODUCTION

Approximately one-third of all orchid species do not provide floral rewards, and rely on various forms of deception for pollination (Dafni 1984; Ackerman 1986). This presents a major evolutionary conundrum, as floral rewards, such as nectar, have been shown in studies with other plants to increase the amount of pollen deposited and removed per visit, as well as the number of flowers probed per plant (cf. Mitchell & Waser 1992; Mitchell 1993; Burd 1995). Indeed, broad surveys reveal that rewarding orchids usually have higher levels of pollination and fruiting success than their deceptive counterparts (Gill 1989; Johnson & Bond 1997; Neiland & Wilcock 1998).

Many authors who have discussed the evolution of deception in orchids have speculated that a high cost of nectar production could have favoured nectarless mutants (Boyden 1982; Ackerman 1986). The main problems with this idea are that reproduction in many orchids is pollen-limited rather than resource-limited, even over a lifetime (Calvo 1993), that it fails to explain why more orchids do not produce very small (and presumably inexpensive) quantities of nectar (Ackerman *et al.* 1994) and that it does not explain why deception should occur mainly in orchids.

Alternative hypotheses for the evolution of deception relate deception to the occurrence of pollinia in the Orchidaceae. Orchid pollen is packaged into pollinia, which attach to pollinators by means of a sticky viscidium, and thus the entire pollen complement of most orchid flowers can be removed in a single pollinator visit. Plants with granular pollen, however, require repeated visits for pollen removal. Harder (2000) argued that deception is viable in orchids because the male function is not compromised as much by low rates of visitation as it is in plants with granular pollen. It was recently proposed that deception might actually increase the likelihood of pollinarium removal from flowers (Smithson & Gigord 2001). Support for this rather counterintuitive idea came from a reduction in pollinarium removal from flowers of *Barlia robertiana* following experimental supplementation with nectar (Smithson & Gigord 2001). Other studies have indicated that pollinarium removal may be increased by the addition of nectar (Johnson & Nilsson 1999) or show no clear effect (Smithson 2002).

Understanding the adaptive significance of deception in plants requires a consideration of both female and male functions, including the fate of pollen removed from flowers. Dressler (1981) proposed that deception serves to limit the number of flowers probed on a plant by each pollinator, and thus reduces geitonogamy (transfer of pollen among flowers on the same plant; see also Dafni & Ivri 1979). Geitonogamous self-pollination compromises female function through inbreeding depression and male function by reducing the amount of pollen available for

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export to other plants (a process known as pollen discounting; cf. de Jong *et al.* 1993; Barrett & Harder 1996). Johnson & Nilsson (1999) argued that limited carryover of orchid pollinia from flower to flower might render orchids particularly prone to the negative consequences of geitonogamy. They showed that the experimental addition of nectar to the flowers of *Orchis mascula* and *Anacamptis morio* resulted in significant increases in the number of flowers probed by each pollinator as well as the total time spent on plants. By also obtaining data on pollen carryover for *O. mascula*, they were able to model the predicted depositions of self-pollen and outcross-pollen on plants when nectar is present or absent in flowers. However, they did not obtain empirical evidence to support their prediction that nectar production would result in higher levels of geitonogamy.

Another mechanism that may reduce geitonogamy in orchids is pollinarium bending (Darwin 1877; Johnson & Edwards 2000). After withdrawal from a flower, pollinaria of many orchid species undergo a bending movement, resulting in a time delay before the pollinium assumes a position from which it can strike a stigma (Johnson & Edwards 2000). Darwin (1877) considered pollinarium bending to be a 'beautiful contrivance' that reduces intrafloral selfing and geitonogamy.

Although deceptive pollination systems and pollinarium bending are traits that are present in many orchids, their consequences for geitonogamous self-pollination have not been demonstrated empirically. Smithson (2002) found that the number of flowers receiving self-pollen was several-fold higher in some nectar-supplemented plants of *A. morio* in southern Europe, but this result was statistically inconclusive owing to a small sample size.

Direct tracking of the fate of pollen in plant populations presents a formidable methodological challenge. Peakall (1989) introduced histochemical staining as a way of tracking the flow of orchid pollen in populations, and estimated levels of geitonogamy from the presence or absence of stained pollen on self-stigmas of several Australian orchid species (Peakall 1989; Peakall & Beattie 1996). As it is only the pollinium that is stained and not the viscidium (the sticky structure that attaches to pollinators), staining has no effect on the removal of pollinaria from flowers (Peakall 1989; this study). However, to the best of the authors' knowledge, there has been no previous investigation of whether staining, by modifying the cohesiveness of pollinia or the adhesion of pollen to stigmas, could influence pollen carryover.

We investigated the effects of nectar addition on pollen fates in the non-rewarding orchid *A. morio*. Using the staining method of Peakall (1989) to label the pollen of *A. morio*, we carried out experiments designed to address the following specific questions: (i) would nectar production affect patterns of pollen carryover (the fraction of a pollinarium carried over from flower to flower); (ii) would nectar production affect pollinarium removal from flowers and inflorescences; (iii) would nectar production lead to increased geitonogamous self-pollination; (iv) does pollinarium bending effectively reduce geitonogamy; and (v) how would the overall rate of self-pollination and the amount of pollen exported be affected by a mutation for nectar production? We also carried out preliminary experiments to test whether the transfer properties of stained pollen differ from those of unstained pollen.

2. MATERIAL AND METHODS

(a) *The study system*

Anacamptis morio (L.) Bateman, Pridgeon & M. W. Chase (syn. *Orchis morio* L.) is a spring-flowering bee-pollinated orchid with a wide distribution in Europe. It typically produces a single inflorescence with approximately eight flowers, each with two pollinaria. Each pollinarium comprises a sticky viscidium, a slender connective caudicle and a sectile pollinium consisting of *ca.* 123.1 ± 14.8 pollen massulae ($n = 12$). Pollen massulae break away from the pollinium individually or in clumps when adhering to the mucilage on the stigma. Thus several flowers can potentially be pollinated by a single sectile pollinium.

Anacamptis morio is self-compatible, but depends on pollinators for fruit set (Nilsson 1984). In Sweden, the species is pollinated primarily by queen bumble-bees (Nilsson 1984). The purple-pink flowers have spurs that do not contain nectar and instead rely on a conspicuous display and sweet scent to elicit exploratory visits from food-seeking bees. This system, termed 'generalized food deception', is found in most European *Orchis* and *Anacamptis* species (Nilsson 1992). Visits are typically brief and made to only one or two flowers on an inflorescence (Nilsson 1984; Johnson & Nilsson 1999; Smithson 2002). Although the flowers do not mimic those of any other rewarding species in the community, the plants experience greater reproductive success when growing in areas rich in 'magnet' plants that provide nectar for bumble-bees (Johnson *et al.* 2003).

The study was conducted during May–June 2002 on the Baltic island of Öland off the southeast coast of Sweden. Field experiments took place in the immediate vicinity of the Ecological Field Station of Uppsala University at Skogsby in a large *A. morio* population consisting of several thousand individuals. Queens of the bumble-bee *Bombus lapidarius* are the most important pollinators of *A. morio* at this site (Nilsson 1984).

(b) *Pollinator behaviour and pollen fate*

To establish whether nectar production would increase the number of flowers probed, the time spent by pollinators on inflorescences, pollinarium removal and the rate of geitonogamous self-pollination, we compared the behaviour of pollinators and the pollen transfer dynamics on unmanipulated and nectar-enriched inflorescences. Each flower on nectar-enriched inflorescences had 2 µl of 25% sucrose solution injected into the tip of the spur by means of a 10 µl microsyringe. In addition, 1–2 µl of histochemical stain was injected into each of the anther sacs to colour-label the pollen. Stains used and their concentrations were fast green (1%) and gentian violet (premixed medicinal preparation—Alpha) added to unmanipulated inflorescences, and neutral red (1%) or rhodamine B (0.2%) added to nectar-enriched inflorescences.

Unmanipulated and nectar-enriched inflorescences were presented individually to foraging *B. lapidarius* queens at the end of a 2 m long cane, the so-called bee-interview technique (cf. Thomson 1988; Johnson & Nilsson 1999). The end of the cane was placed in the grass *ca.* 20 cm from a bee feeding on nectar plants, and, in *ca.* 10–30% of cases, bees chose to alight on the orchid inflorescence. This method allowed us to record the behaviour of pollinators, which are otherwise extremely difficult to observe on orchids (cf. Nilsson 1984). Flower number per inflorescence did not differ significantly between the two

treatments (8.6 ± 1.9, $n = 43$, for unmanipulated versus 7.9 ± 2.0, $n = 49$, for nectar-enriched; $t = 1.2$, $p = 0.24$). We recorded the number of flowers probed, the time taken per probe and the position of the probed flowers on the inflorescences using a microcasette recorder. Presentations were made to bees foraging on the flowers of *Allium schoenoprasum*, an important nectar plant that grows in association with *A. morio* in the meadows around the field station. While it is almost impossible to determine whether bees are carrying unstained pollinaria as they approach an inflorescence, we were able to avoid bees that carried the conspicuous stained pollinaria from previous visits to the experimental inflorescences. This prevented us from confusing self-pollen and outcross-pollen stained the same colour, and also prevented resampling of the same insects. At the end of each foraging bout the inflorescence was stored for later examination of the stigmas under a dissecting microscope.

In an independent experiment, we translocated an additional 22 pairs of unmanipulated and nectar-enriched inflorescences into the field for a 48 h period. Flower number per inflorescence did not differ significantly between the two treatments (7.3 for unmanipulated versus 7.0 for nectar-enriched; paired $t = 0.4$, $p = 0.72$). These inflorescences were placed with the cut base of the stem in a small vial filled with moist florist's foam to prevent wilting. To minimize the possibility that pollen imported from a plant labelled with the same stain as the recipient would be mistaken for self-pollen, inflorescences stained with the same colour were separated by at least 20 m in a dense natural population of *A. morio*. At the end of the 48 h period, flowers were examined under a dissecting microscope. In contrast to the presentation experiment described above, we did not monitor pollinator visitation behaviour in the translocation experiment. Individual inflorescences may have been visited more than once during the experiment.

For each inflorescence in the presentation and translocation experiments, we determined the number of self-pollen massulae (labelled with the same stain as the recipient plant) and the number of outcross massulae (unstained, or labelled with a different stain) on the stigmas. We also recorded how many of the pollinaria had been removed from the flowers. We compared unmanipulated controls with nectar-enriched plants in terms of the absolute numbers of pollinaria removed and pollen massulae deposited, as well as the fraction of the removed pollen that was deposited on self-stigmas. For the presentation experiments, we also compared the numbers of pollinaria removed per flower visit on unmanipulated and nectar-enriched inflorescences.

To estimate the rate of self-pollination in the population, we combined data on the fraction of removed pollen that was deposited on self-stigmas with an estimate of the overall proportion of removed pollen that is deposited on stigmas, 'the pollen transfer efficiency'. We quantified pollen transfer efficiency as

$$\mathrm{PTE} = M_s/(P_r \times M_n), \tag{2.1}$$

where M_s is the mean number of pollen massulae deposited per stigma, P_r is the mean number of pollinaria removed per flower and M_n is the number of massulae per pollinium. We estimated M_s and P_r by scoring the number of massulae deposited and the number of pollinaria removed, respectively, in a sample of 113 flowers, each picked haphazardly from a different plant in the population. This enabled us to calculate the fraction of removed pollen involved in geitonogamous self-pollination, F_s, as

$$F_s = F_g/\mathrm{PTE}, \tag{2.2}$$

where F_g is the fraction of removed stained massulae deposited on self-stigmas. This is based on the assumption that geitonogamy involves complete discounting of the pollen export, i.e. that overall PTE is not affected by changes in geitonogamy (cf. Lloyd 1992). Finally, the actual number of pollen massulae exported from a plant to other plants in the population, M_e, can be calculated as

$$M_e = P_p \times M_n \times \mathrm{PTE} \times (1 - F_s), \tag{2.3}$$

where P_p is the number of pollinaria removed from a plant.

(c) *Pollinarium bending and self-pollination*

Pollinarium bending in *A. morio* is completed in *ca.* 30 s (Johnson & Nilsson 1999), but simulations with dead bees positioned in flowers have shown that pollinia reach a position where they may contact the stigma 18–20 s after removal from a flower (S. D. Johnson, C. I. Peter and J. Xgren, unpublished data). To determine whether pollinarium bending delays the onset of geitonogamy, we compared the frequency of visits resulting in geitonogamous self-pollination among visits lasting more and less than 18 s.

(d) *Pollen carryover*

Our experimental design is based on the assumption that removal and transfer of labelled pollen reflects that of unstained pollen. Moreover, interpretation of the results depends on whether pollen removal and carryover are similar in nectar-enriched and control inflorescences. To determine whether the experimental addition of nectar or the labelling of pollen with histochemical stains affects pollen carryover in *A. morio*, we allowed bumble-bees with a pair of freshly affixed pollinaria to visit virgin emasculated inflorescences and counted the number of pollen massulae deposited on each flower in the sequence. The experiments were conducted in the greenhouse at the Ecological Field Station with *B. lapidarius* queens.

After removing two pollinaria from a single flower, bees were left for a few minutes to allow the pollinaria time to undergo their characteristic bending movement. To establish the effect of nectar production on pollen carryover, we allowed the bumble-bees to visit either a sequence of emasculated control flowers or a sequence of emasculated flowers that had had 2 µl of 25% sucrose solution injected into the tip of the spur (the same volume and concentration as we used in the field experiments). To determine the effects of pollen staining on carryover, bumble-bees carrying two freshly affixed stained pollinaria were allowed to visit a sequence of virgin emasculated non-enriched flowers using the same procedure. For these runs, we injected 1–2 µl of histochemical stain into the anther of a flower, and after 30 min (to allow the pollinia to dry) we presented the flower to a bumble-bee. Bees always removed both pollinaria from these stained flowers on their first visit, regardless of the type of stain that had been applied to the anther. We repeated these experiments for each of the four stains at the same concentrations as we used in the field experiments (i.e. fast green, gentian violet, neutral red, rhodamine B).

After each visitation sequence in the carryover experiments described above, individual visited flowers were removed, numbered and examined under a dissecting microscope to allow deposited massulae to be counted. We carried out between three and six runs, each consisting of visits to a sequence of 10 flowers, for each treatment. Individual bees were used for only one run within a treatment, but reused in a random sequence for runs in different treatments, in which case previous pollinaria were

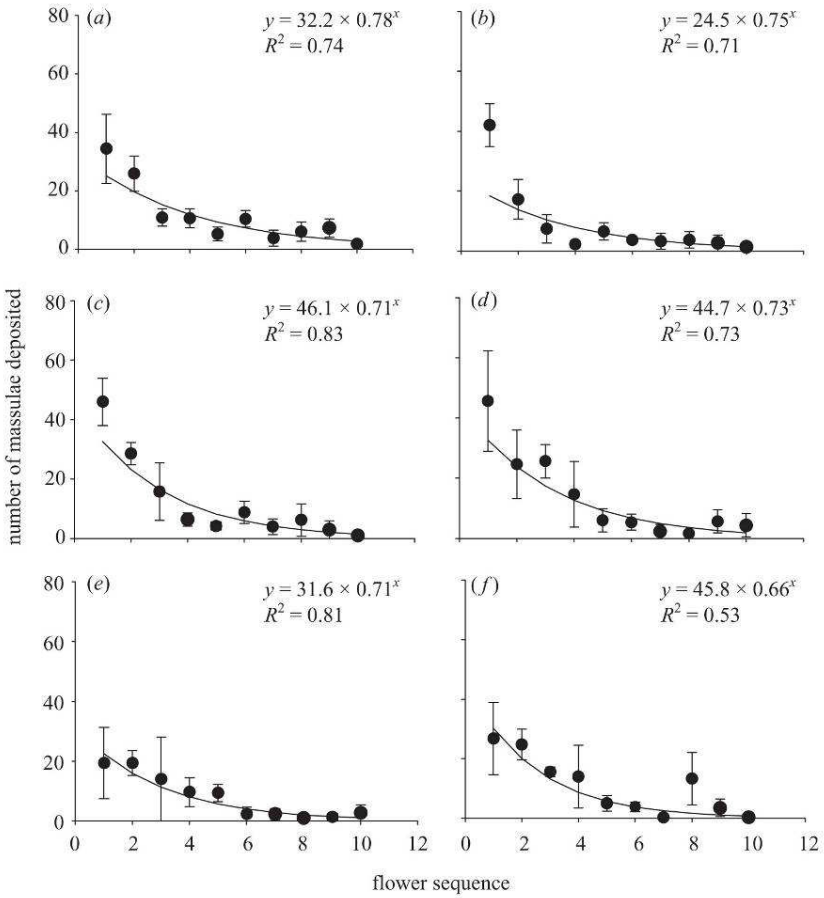

Figure 1. Pollen carryover in *Anacamptis morio*. Data represent the mean (± s.e.) numbers of pollen massulae deposited over a sequence of virgin emasculated flowers visited consecutively by queen bumble-bees (*Bombus lapidarius*) carrying two pollinaria: (*a*) non-rewarding flowers, (*b*) recipient flowers containing nectar, (*c–f*) non-rewarding flowers that received depositions from pollinaria stained with each of the four coloured histochemicals used in the field experiments: (*c*) rhodamine B, (*d*) neutral red, (*e*) gentian violet and (*f*) fast green.

removed from the bees with forceps at the commencement of a new run.

For each treatment, we fitted a simple linear regression and an exponential decay function to the observed relationship between the mean number of massulae deposited and the position of the flower in the visitation sequence. In the exponential-decay model, $y = ab^x$, b is the carryover fraction, and $(1 - b)$ is thus the fraction of donor pollen removed from the pollinator and deposited on the stigma at each visit, assuming no pollen losses in transit and a constant carryover fraction (cf. Morris *et al.* 1994). We used ANCOVA to examine whether the slope of this relationship was affected by nectar supplementation and whether it varied among staining treatments (analysis based on the linearized function $\ln y = \ln a + x \ln b$).

The mean numbers of pollen massulae deposited on a sequence of flowers from a pair of pollinaria fitted an exponential-decay model better than they did a linear model (unmanipulated control inflorescences: exponential $R^2 = 0.74$; linear $R^2 = 0.63$). The slope of the fitted curve (exponential-decay model) did not differ among treatments (interaction in ANCOVA: $F_{5,48} = 0.5$, $p = 0.79$). This suggests that nectar addition does not markedly affect pollen carryover, and that the carryover properties of stained and unstained pollen are approximately the same. According to the fitted curves, between 22% and 34% of the pollen load is deposited on each sequential flower in *A. morio* (figure 1).

3. RESULTS

(a) *Effects of nectar on pollinator behaviour and pollen fate: presentation experiment*

Addition of nectar to floral spurs of *A. morio* affected pollinator behaviour considerably. The number of flowers probed by bumble-bees was 2.3 times higher, the time spent on an inflorescence was 5.3 times longer and the time spent probing individual flowers was 2.2 times longer on nectar-enriched than on control inflorescences (figure 2*a–c*). When on nectar-enriched inflorescences, bumble-bees removed a significantly higher proportion of pollinaria from flowers that were probed then they did on control inflorescences (figure 2*d*).

In the presentation experiment, nectar addition affected both pollen removal and the rate of self-pollination. Bumble-bees removed 3.8 times more pollinaria from nectar-enriched than from control inflorescences (figure 2*e*). Approximately 4% of the pollen removed from nectar-

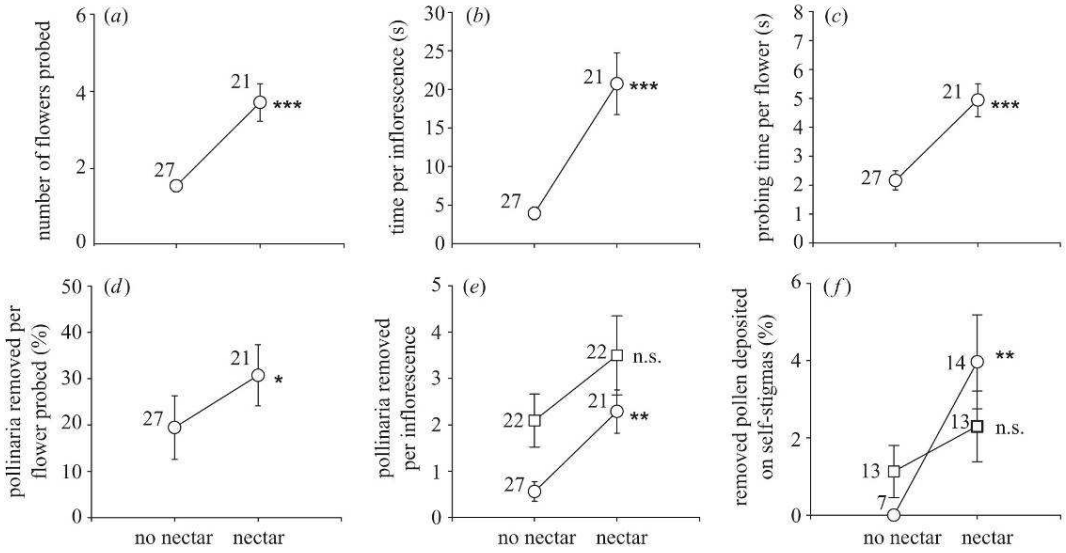

Figure 2. The effects of nectar addition to flowers of *Anacamptis morio* on pollinator behaviour (*a–c*) and pollen fates (*d–f*). Symbols (circles, presentations; squares, translocations) indicate means (± s.e.) and are flanked by sample sizes and asterisks indicating statistical significance ($^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$) of two-sample *t*-tests. See § 3 for further statistical analyses.

enriched inflorescences was deposited on self-stigmas, while no self-pollination was observed in control inflorescences (figure 2*f*). These differences were statistically significant (two-sample *t*-tests: $p < 0.01$).

(b) *Effects of nectar on pollen fate: translocation experiment*

In the translocation experiment, the effects of nectar addition tended to be weaker and the variances higher than in the presentation experiment. Bumble-bees removed approximately 1.7 times more pollinaria, and the proportion of pollen deposited on self-stigmas was almost twice as high for nectar-enriched than for control inflorescences (figure 2*e,f*), but these differences were not statistically significant (two-sample *t*-tests: $p > 0.05$).

In combined analyses (two-way ANOVA with experimental procedure and nectar addition as fixed factors), nectar addition had a significant overall effect on pollinaria removal ($F_{1,88} = 8.04$, $p = 0.006$) and on the proportion of removed pollen deposited on self-stigmas ($F_{1,43} = 6.49$, $p = 0.014$). Experimental procedure (presentation versus translocation) had a significant overall effect on pollinaria removal ($F_{1,88} = 6.16$, $p = 0.015$), but not on the rate of self-pollination ($F_{1,43} = 0.07$, $p = 0.79$). The interactions of experimental procedure and nectar addition were not significant for either pollinaria removal ($F_{1,88} = 0.08$, $p = 0.77$) or rate of self-pollination ($F_{1,43} = 1.92$, $p = 0.17$), indicating that the effects of nectar addition on these response variables were similar in both experiments (see figure 2*e,f*).

(c) *Pollinarium bending and self-pollination*

The probability of geitonogamy was related to the time spent on the inflorescence, as predicted by the pollinarium bending time. The proportion of visits to inflorescences that resulted in self-deposition was markedly lower among the 37 visits that were shorter than 18 s (5.4%) than among the 11 visits longer than 18 s (63.6%; $\chi^2 = 10.6$, $p = 0.001$; figure 3*b*). The near absence of geitonogamy for those visits shorter than 18 s is remarkable given that in most of these the pollinator probed several flowers on a plant (figure 3*a*).

(d) *Pollen transfer efficiency*

In the study population, 0.75 ± 0.91 pollinaria were removed per flower (mean ± s.d.) and the mean stigmatic pollen load was 7.5 ± 18.1 massulae ($n = 117$). Given that each pollinium contains 123.1 massulae (see § 2a), the overall percentage of removed pollen that is deposited on stigmas, the 'pollen transfer efficiency', is 8.1%.

4. DISCUSSION

The results of this study support the hypothesis that deception in orchids reduces geitonogamous self-pollination (cf. Dafni & Ivri 1979; Dressler 1981; Johnson & Nilsson 1999; Johnson 2000). They also confirm Darwin's original prediction that the gradual bending of pollinaria after withdrawal from an orchid flower is a mechanism that reduces the occurrence of intra-floral and geitonogamous self-pollination (figure 3). In *A. morio*, lack of nectar and pollinarium bending together ensure that self-depositions occur rarely (figure 2*f*), and the mating system is thus likely to be characterized by high levels of outcrossing.

Our data do not support the pollinaria-removal hypothesis for the evolution of deception proposed by Smithson & Gigord (2001). Addition of nectar to spurs of *A. morio* resulted in significant increases in both the total number of pollinaria removed from inflorescences and the rate of removal of pollinaria from individual probed flowers (figure 2*d,e*). The pollinaria-removal hypothesis was also not supported by previous studies of *A. morio*, which showed either a significant increase in pollinaria removal

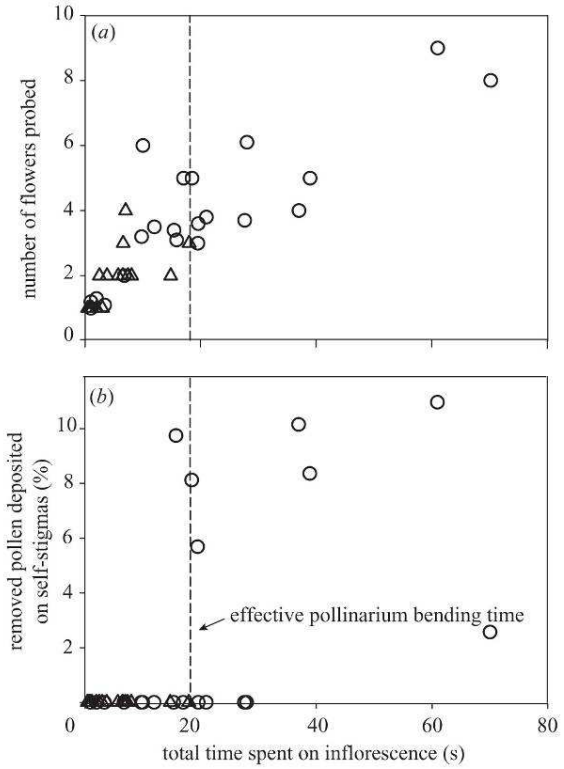

Figure 3. The relationship between the time that bumblebees spend on inflorescences of *Anacamptis morio* and (*a*) the number of flowers probed and (*b*) the percentage of removed pollen that is deposited on self-stigmas. The time taken for freshly removed pollinaria to bend into a position from which they can strike a stigma is indicated by the dashed line. Symbols: triangles, non-rewarding inflorescences; circles, rewarding inflorescences.

with nectar addition in one of two populations (Johnson & Nilsson 1999) or non-significant effects on overall pollinaria removal from inflorescences (Smithson 2002).

The presentation and translocation experiments used in this study yielded similar results for overall pollen fate (figure 2*e*,*f*). The main difference between the two experiments was that the presentations involved a single visit to each inflorescence and thus simulated high levels of nectar replenishment in nectar-producing plants, while the translocations allowed multiple visits to inflorescences without replenishment of nectar. If nectar replenishment in visited flowers is slow or does not occur at all, then pollinators would regularly encounter empty flowers on nectar-producing mutants and the evolution of nectar production would be expected to have a less marked influence on pollen fates. The non-significant interaction between nectar addition and experiment type in the two-way ANOVA suggests that nectar replenishment rate either has no effect on pollen fates or has an effect that would be detectable only with a larger sample size. Additional experiments are required to examine fully the effect of nectar-production schedules on pollinator behaviour and pollen transfer.

Although the pollen deposited on self-stigmas of nectar-supplemented plants made up a small percentage (3.2%) of the pollen removed from flowers, it is nevertheless significant in relation to the 8.1% of removed pollen that reaches stigmas in this population. In the absence of pollen discounting, self-depositions would comprise 28.3% of the removed pollen deposited. However, a more realistic scenario is that geitonogamous self-pollination is discounted completely against the exportable fraction of pollen (cf. Lloyd 1992; Barrett & Harder 1996). The exponential decline in the amounts of pollen deposited on consecutive stigmas (figure 1) illustrates how pollen deposited on flowers of the same plant might compromise pollen export. Based on pooled data from the two field experiments, we calculate that self-pollination in nectar-producing mutants could involve as much as 39.5% of the removed pollen that is deposited on stigmas (equation (2.2)). By contrast, self-pollination in deceptive plants would involve just 8.6% of the removed pollen deposited (equation (2.2)).

A mutation for nectar production in *A. morio* would result in substantially increased levels of pollinaria removal from inflorescences (figure 2*d*; Johnson & Nilsson 1999). However, the efficiency of pollen export in these mutants is likely to be diminished as a consequence of greater levels of geitonogamy (figure 2*f*). The male fitness outcome would depend heavily on the visitation rate by pollinators. This can be illustrated by considering two scenarios for typical plants with eight flowers, the first in which pollinators are limiting and each plant is visited just once, and the second in which pollinators are abundant and remove all of the pollinaria from plants. In the first scenario, bees would remove 2.3 pollinaria from nectar-producing plants and 0.6 pollinaria from deceptive plants, as was observed in our presentation experiment (figure 2*d*). Assuming complete pollen discounting, i.e. a constant overall pollen-deposition fraction, 13.9 massulae would be exported by rewarding plants versus 5.6 massulae by deceptive plants (equation (2.3)). In the second scenario, 96.4 massulae would be exported by rewarding plants versus 149.2 massulae by deceptive plants (equation (2.3)). Thus overall male fitness is likely to be lower in deceptive than in rewarding plants when pollinators are scarce, but higher when pollinators are abundant.

The effects of nectar on female reproductive success were harder to gauge in this study because the majority of bees that we used in the presentation experiments did not arrive with pollinaria and thus seldom cross-pollinated the experimental flowers. Our carryover data, however, indicate that, while nectar would not have a significant effect on pollen deposition per flower probed by bees already carrying pollinaria (figure 1), the greater number of flowers probed (figure 2*a*) would translate into greater overall pollination success. Earlier experiments detected a significant increase in pollen receipt for nectar-supplemented plants in an *A. morio* population with low rates of visitation by pollinators (Johnson & Nilsson 1999). When pollinators are scarce, nectar production would be expected to lead to higher levels of fruit set with some of the fruits arising from geitonogamous self-pollination. When pollinators are common enough for most flowers to set fruit, selection through the female component of fitness will favour deception if the resultant increase in outcrossing leads to higher fruit quality. There is strong inbreeding depression for fruit quality in *A. morio*. Embryos of

A. morio arising from self-fertilization are twice as likely to abort in the early stages of development as are embryos arising from cross-fertilization (S. D. Johnson and J. Ågren, unpublished data). Genetic load would be expected to accumulate in deceptive species owing to the rarity of pollinator-mediated self-pollination, although quantitative data to evaluate this hypothesis are still rare (Ferdy *et al.* 2001; Wallace 2003). In addition, the high costs of fruit production (cf. Ackerman & Montalvo 1990) might offset some of the advantages of increased fruit production in nectar-producing mutants when pollinators are scarce.

A combination of reduced outcrossing opportunities and high levels of inbreeding depression in nectar-producing mutants may explain the evolutionary maintenance of deception in a species such as *A. morio* that is relatively well visited, with 20–50% of flowers having either pollen deposited or pollinaria removed in most populations at our study site (Johnson *et al.* 2003). It is more difficult to understand how selection could maintain deception in the many orchids that consistently have fewer than 10% of their flowers visited by pollinators (cf. Gill 1989; Neiland & Wilcock 1998). Further studies of the relationships between reward production, pollen-transfer dynamics and inbreeding depression are clearly needed for a more complete understanding of the evolution of floral deception in these species.

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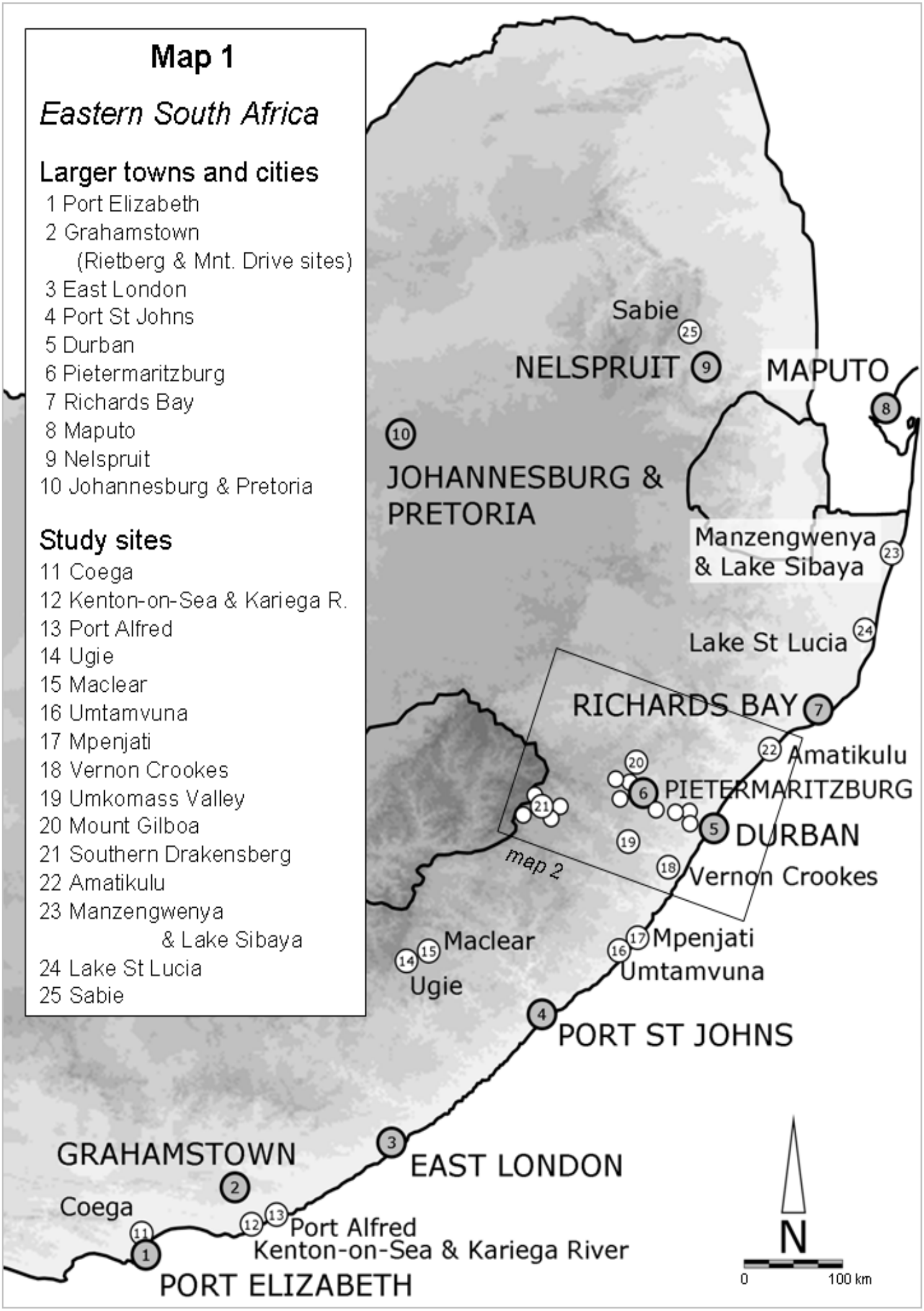
Map 1
Eastern South Africa
Larger towns and cities
1 Port Elizabeth
2 Grahamstown
(Rietberg & Mnt. Drive sites)
3 East London
4 Port St Johns
5 Durban
6 Pietermaritzburg
7 Richards Bay
8 Maputo
9 Nelspruit
10 Johannesburg & Pretoria
Study sites
11 Coega
12 Kenton-on-Sea & Kariega R.
13 Port Alfred
14 Ugie
15 Maclear
16 Umtamvuna
17 Mpenjati
18 Vernon Crookes
19 Umkomass Valley
20 Mount Gilboa
21 Southern Drakensberg
22 Amatikulu
23 Manzengwenya
& Lake Sibaya
24 Lake St Lucia
25 Sabie
Sabie
NELSPRUIT
MAPUTO
JOHANNESBURG &
PRETORIA
Manzengwenya
& Lake Sibaya
Lake St Lucia
RICHARDS BAY
Amatikulu
PIETERMARITZBURG
DURBAN
Vernon Crookes
map 2
Maclear
Ugie
Mpenjati
Umtamvuna
PORT ST JOHNS
GRAHAMSTOWN
EAST LONDON
Coega
Port Alfred
Kenton-on-Sea & Kariega River
PORT ELIZABETH
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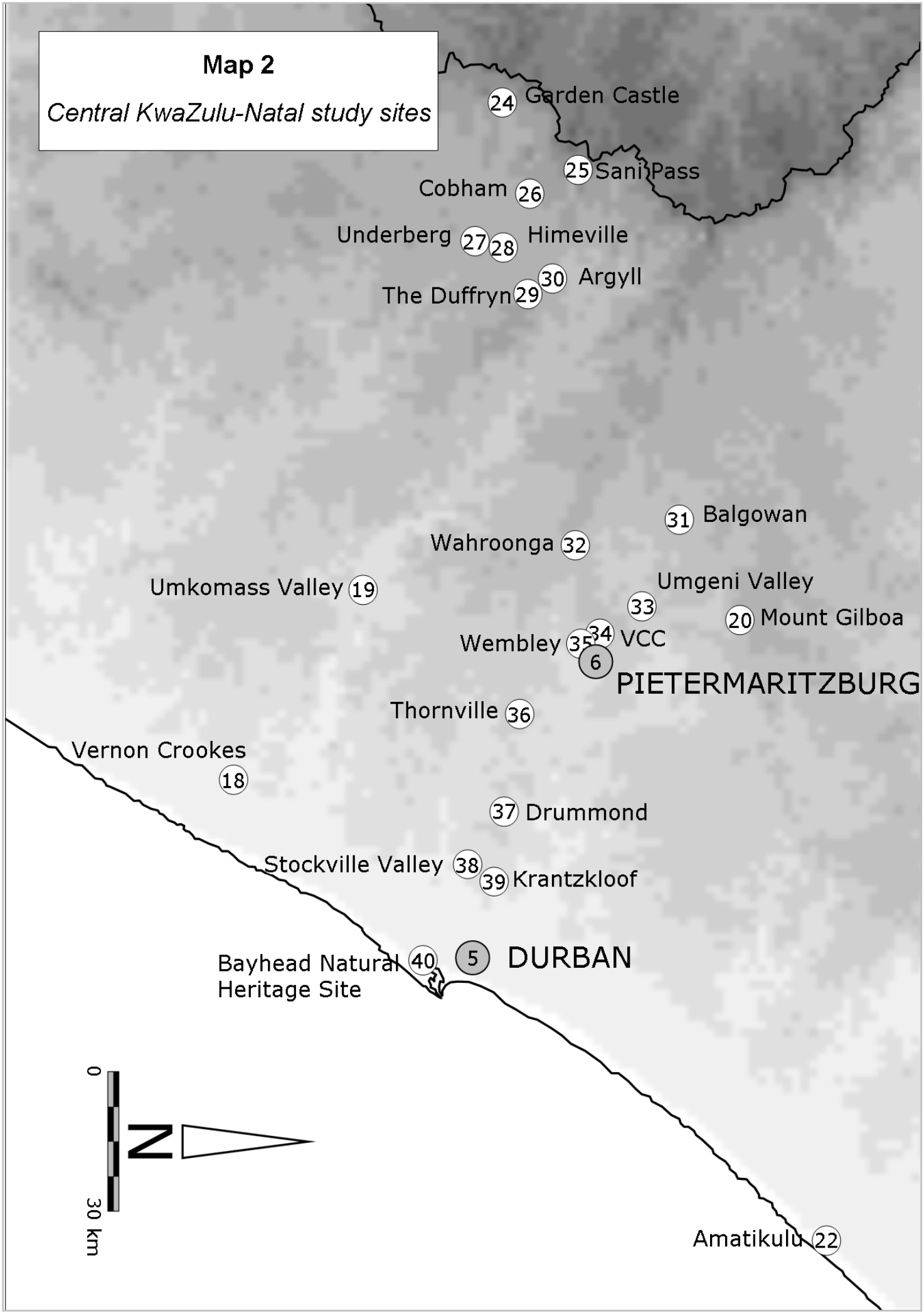
Map 2
Central KwaZulu-Natal study sites
24 Garden Castle
25 Sani Pass
Cobham 26
Underberg 27
28 Himeville
30 Argyll
The Duffryn 29
31 Balgowan
Wahroonga 32
Umkomass Valley 19
Umgeni Valley
33
20 Mount Gilboa
Wembley 35
34 VCC
6
PIETERMARITZBURG
Thornville 36
Vernon Crookes
18
37 Drummond
Stockville Valley 38
39 Krantzkloof
Bayhead Natural Heritage Site
40
5 DURBAN
0
30 km
N
Amatikulu 22

All done!