

## A pollinator shift explains floral divergence in an orchid species complex in South Africa

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• **Background and Aims** 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. The current circumscription of the food-deceptive southern African orchid *Eulophia parviflora* encompasses a highly variable monophyletic species complex. In this study, two forms were identified within this complex that differ in distribution, floral morphology, scent chemistry and phenology, and a test was made of whether these differences represent adaptations for different pollinators.

• **Methods and Results** Multivariate analysis of floral and vegetative traits revealed that there are at least two discrete morphological forms in the species complex. Field observations revealed that each form is pollinated by a different insect species, and thus represent distinct ecotypes. The early-flowering coastal form which has long spurs and floral scent dominated by sesquiterpene compounds is pollinated exclusively by the long-tongued bee *Amegilla fallax* (Apidae, Anthophorinae), while the late-flowering inland form with short spurs and floral scent dominated by benzenoid compounds 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 initially thought likely that divergence occurred across a geographical pollinator gradient, plants of the long-spurred form were effectively pollinated when transplanted to an inland locality outside the natural coastal range of this form. 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.

• **Conclusions** This study identifies a shift between bee and beetle pollination as the main driver of floral divergence in an orchid species complex. Floral scent and spur length appear to be key traits in mediating this evolutionary transition.

**Key words:** Grant–Stebbins model, pollinator-driven speciation, pollination ecotypes, scent, *Eulophia*, Orchidaceae, phenology, beetle pollination, Cetoniinae, bee pollination, Y-maze olfactometer, *Amegilla*.

### 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 can result in divergence of ‘pollination ecotypes’ within a species (Ambruster, 1985; 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 and Hodges, 1999; Bradshaw

and Schemske, 2003). However, by selective modification of some of the most conspicuous traits of plants, pollinators are also responsible for the evolution of 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 (cf. Conner, 2006; Morgan, 2006), as well as macroevolutionary studies that show links between shifts in pollination system and cladogenesis (Givnish and Sytsma, 1997; Johnson *et al.*, 1998; Soltis *et al.*, 2005; Larsen *et al.*, 2008; Forest *et al.*, 2014). 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 and Wyatt, 1990; Johnson, 1997; Johnson and Steiner, 1997; Boyd, 2004; Anderson *et al.*, 2010; Nattero *et al.*, 2010; Boberg *et al.*, 2014; Newman *et al.*, 2014; van der Niet *et al.*, 2014). Flowering phenology is another trait that has been investigated with respect to pollinator shifts among forms within a species (Herrera *et al.*, 2002). There is also some evidence that intraspecific variation in floral scent chemistry can be associated with different pollinators (Pellmyr, 1986; Johnson *et al.*, 2005b; Schlumpberger and Raguso, 2008; Peakall and Whithead, 2014), but this remains poorly documented. It should also be noted that several studies have not found clear evidence that the distribution of floral forms corresponds to a geographical mosaic of pollinators (Robertson and Wyatt, 1990; Herrera *et al.*, 2002, 2006). In some recent studies, geographical variation in floral traits has been attributed to geographical variation in the behaviour of a single pollinator (Ellis and Johnson, 2009; Newman *et al.*, 2012).

Very few of the above-mentioned 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 (Ellis and Johnson, 2009; Newman *et al.*, 2012). Arrays can be assembled under laboratory conditions and presented to captive insects (Galen, 1989; der Jager and Ellis, 2014) or arranged in the field by means of transplant experiments (Robertson and Wyatt, 1990; Newman *et al.*, 2012; Sun *et al.*, 2014) or by manipulating flowers within a population (Johnson and Steiner, 1997).

While investigating the pollination biology of *Eulophia parviflora*, we encountered two forms that appeared to differ in floral morphology, floral fragrance and flowering time. We hypothesized that these differences reflect adaptations to different pollinators, and thus constitute pollination ecotypes. To test this hypothesis, we tested the following predictions that emerge from it: (1) morphology, scent chemistry and flowering times would be quantitatively different between the forms; (2) suites of floral traits of each form would show integration into syndromes and be geographically structured; (3) pollinators would differ between the two forms; (4) pollinators would discriminate between the two forms when offered a choice; and (5) floral traits that differ among forms would influence the effectiveness of pollinators.

## MATERIALS AND METHODS

### Study species

*Eulophia* is a large genus of terrestrial orchid found predominantly in Africa. They have sympodial growth with sub-terranean tubers that produce a new vegetative shoot and an inflorescence from the base of the vegetative shoot each year. All taxa of *Eulophia* that we have examined, including the two forms described here, are deceptive and do not reward their pollinators (Peter and Johnson, 2013), although there is evidence that two pantropical species may reward their pollinators (Singer and Cocucci, 1997; Jürgens *et al.*, 2009).

*Eulophia parviflora* (Fig. 1), described by Hall (1965, p. 149) as a ‘rather variable species’, occurs in grasslands of the eastern parts of South Africa (Fig. 2). While investigating the pollination

biology of this species complex, we recognized two distinct forms. One form has tall dense inflorescences with large numbers of non-resupinate flowers and short spurs (Fig. 1A). This form, ‘the short-spurred form’, flowers later in the season, and has relatively well developed vegetative shoots 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. 1D) and an attractive sweet, floral scent. At anthesis, the vegetative shoots rarely have emerged from the ground. Recent phylogenetic analyses of *Eulophia* revealed that both forms currently recognized as *E. parviflora* constitute a clade (Martos *et al.*, 2014).

Both forms of *E. parviflora* have a pair of solid pollinia forming a single pollinarium which is removed as a unit by pollinators. The pollinarium undergoes a bending movement similar to that of *E. streptopetala* (described by Peter and Johnson, 2013) which is thought to limit geitonogamous selfing (Peter and Johnson, 2006b). The pollinaria of these two forms are easily distinguished from those of co-occurring congeners (*E. clitellifera*, *E. clavicornis*, *E. ovalis*, *E. ensata* and *E. foliosa*), all of which have obviously smaller pollinaria.

### 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 Krantzklouf in September 2004, Grahamstown in 2006 and Pietermaritzburg in 2012, and of the long-spurred form in August 2013.

### Morphometric analysis

*Floral and vegetative characters.* To determine if there are morphological discontinuities between the forms, a total of 69 characters were measured for 52 specimens from ten populations and 47 specimens from eight populations of the short- and long-spurred forms, respectively (Supplementary Data Table S1). Missing data accounted for 1.5 % of the characters scored. The majority (61) of these measurements are quantitative characters, with the balance being ratios which describe the shape of floral parts such as petals (Supplementary Data Table S1). Morphological measurements were recorded from living plants or specimens in 70 % ethanol.

Morphological data were analysed using PCA in STATISTICA (StatSoft, 2012). Quantitative data were first log-transformed (Jolicoeur, 1963; Humphries *et al.*, 1981) and a correlation matrix calculated. The first two principle components were plotted against each other. Eigenvalues are given in Supplementary Data Table S2.

### Colour analysis

To determine if the two forms differ in flower colour, spectral reflectance of various flower parts (lateral petals and labella) was 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.

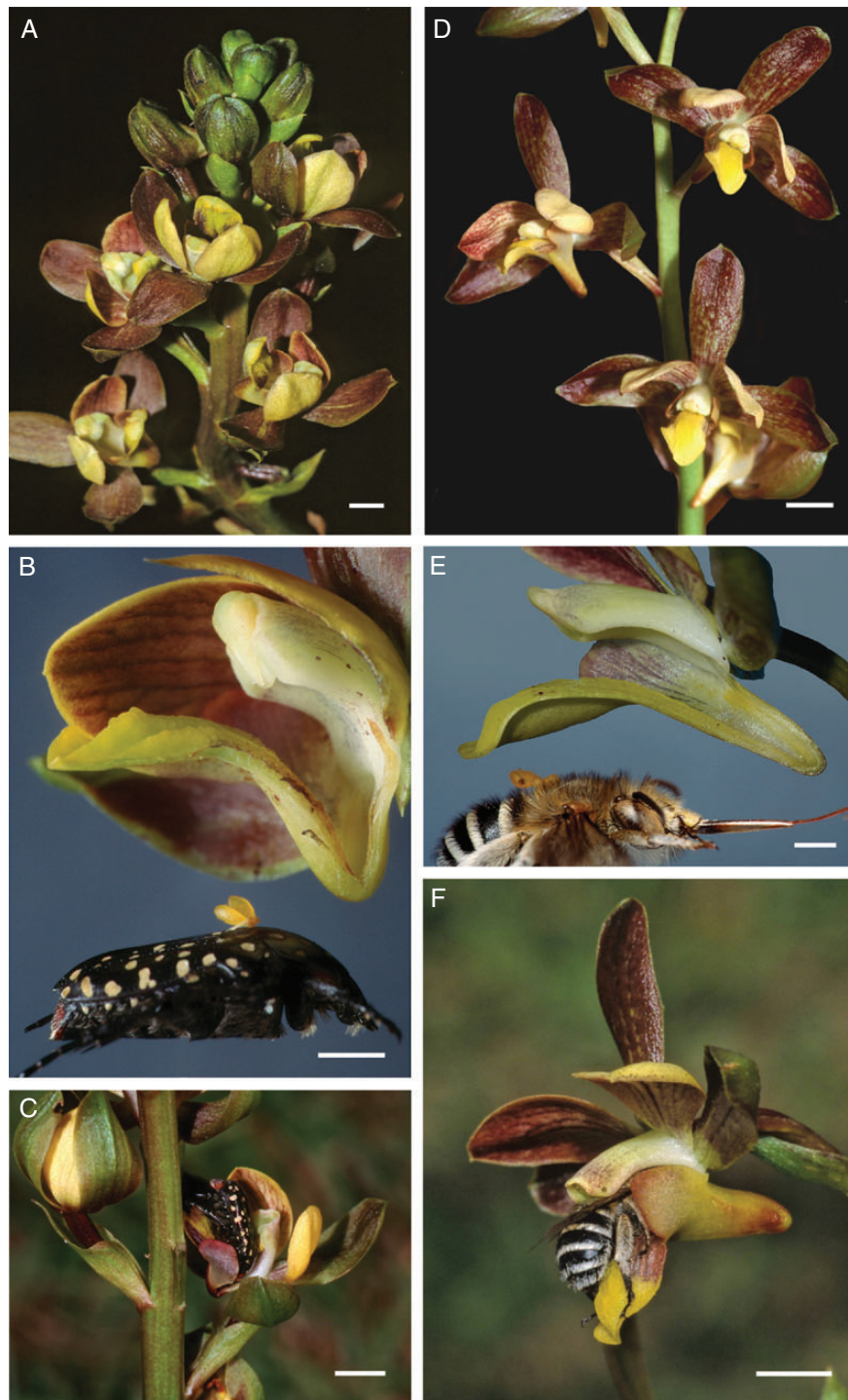


FIG. 1. (A–F) Floral morphology and pollinators of *Eulophia parviflora*. (A) Short-spurred morph. (B) Dissected flower of the short-spurred form showing the short wide spur that accommodates the short blunt head of pollinating *Cyrtothyrea marginalis* beetles. (C) *Cyrtothyrea marginalis* visiting a 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 proboscides of *Amegilla fallax*. (F) *Amegilla fallax* visiting a flower of the long-spurred form. Scale bars = 5 mm, except B and E = 2 mm.

Spectra were summarized using both the Endler segment classification method (Endler, 1990) and the Chittka bee vision model (Chittka, 1992). Given the very different pollinators, including a beetle with unknown visual sensitivity and the presence

of red coloration on the adaxial surface of the lateral petals, colours to which some beetles are known to respond, we selected the Endler (1990) method as being independent of the visual physiology of the insects concerned. We also use the Chittka



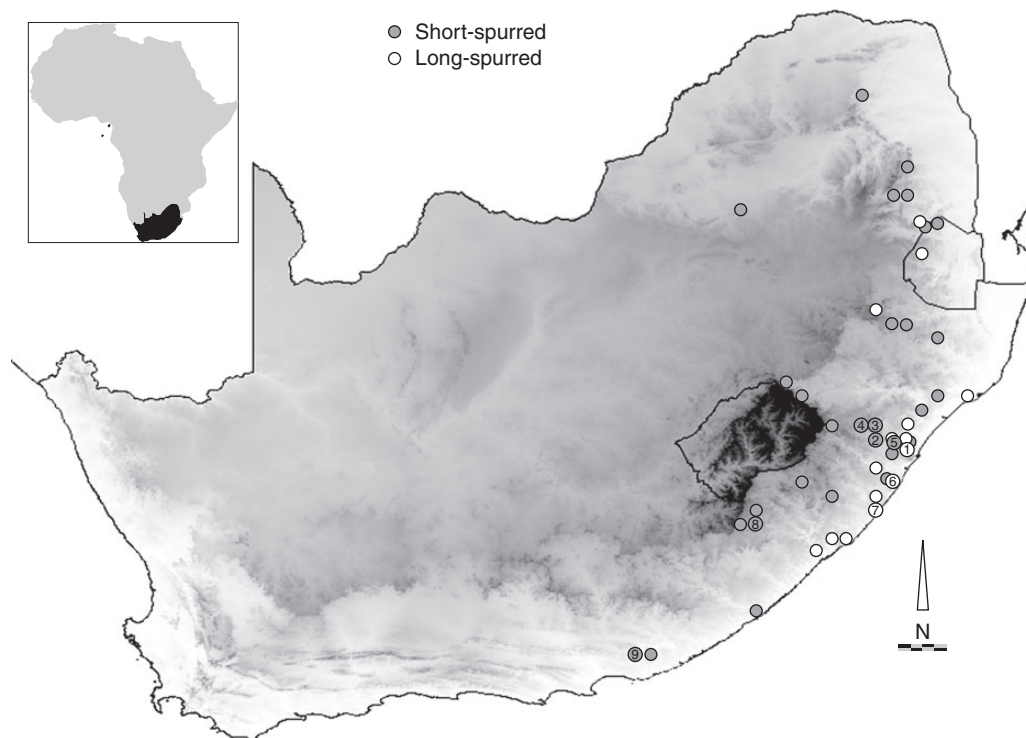


FIG. 2. Distribution of the short- and long-spurred forms (as indicated in the key) of *Eulophia parviflora* in South Africa. Study sites in KwaZulu-Natal include (1) Kloof and Hillcrest [1a, Krantzkloof Nature Reserve, Bridal Road (top and bottom sites); 1b and 1c, Stockville valley (top and bottom respectively); 1d, Galloway Road]; (2) Victoria Country Club and Worlds View on the outskirts of Pietermaritzburg; (3) Umgeni Valley Nature Reserve near Howick; and (4) road verge near Balgowan in the KwaZulu-Natal midlands. Additional observations were made at (5) Drummond; (6) Vernon Crookes nature reserve; and (7) Umtamvuna nature reserve. In the Eastern Cape Province, sites include (8) the outskirts of the town of Maclear and (9) the Rietberg, near Grahamstown. Darker shades of grey represent increasing altitude. Scale bar = 100 km.

(1992) model to summarize these colours in a bee vision model (Chittka and Kevan, 2005).

The Endler (1990) segment classification takes the integral of light reflected from floral parts 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. Colour measurements were recorded from freshly harvested flowers.

#### *Distribution, flowering phenology and pollinator flight times*

Distribution and flowering phenology data were collected from specimens from a variety of herbaria including NU, NH, PRE, BOL, GRA and K. In addition, our field observations were included as equivalent to these herbarium records (each observation representing an opportunity where a single herbarium specimen could have been collected). Localities accurate to at least one minute of latitude and longitude were 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 of *Amegilla*. Distribution data for the two pollinator species are given by Eardley (1994) and Holm and Marais (1992).

In all cases, dates were numbered consecutively, with 1 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 h were spent in the field studying pollinators of the long- and short-spurred forms, respectively. Observations at each site ranged between 1 h at the Maclear site and a total of about 67 h 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 were collected and inspected for pollinaria or viscidia. Where pollinators were observed visiting flowers, the duration of the visits to individual inflorescences was recorded. Voucher specimens are lodged in the Albany Museum and the personal collection of the first author.

#### *Pollinarium reconfiguration*

Pollinaria in most species of *Eulophia* undergo a reconfiguration following removal from the flower. This effectively prevents self-pollination until the reconfiguration is completed,

and there is evidence from surveys of a variety of orchids that the timing of this movement is adapted to exceed the average visit times of specific pollinators (Peter and Johnson, 2006b). Given the hypothesis that these two forms are pollination ecotypes and pollinated by different pollinators, we expected the pollinarium re-configuration times of the two forms to differ. We therefore recorded re-configuration times of one pollinarium per inflorescence for 49 and 81 plants of the short- and long-spurred forms, respectively. Bending times for both forms could not be normalized and were hence compared using the Mann–Whitney U-test.

#### Scent analysis

The scents of the two forms differ to the human nose. We therefore analysed the composition of headspace samples collected from the two forms. Initially, scent samples of three inflorescences of each form were collected in the field (sites of all scent collections are given in Supplementary Data Table S4). Each inflorescence was enclosed in a glass bell jar and headspace air was drawn through a filter containing 3 mg of Porapak for approx. 6 h (at approx.  $2 \text{ L h}^{-1}$ ). Scent compounds were eluted in a 5:1 hexane:acetone mixture and their relative abundances were determined using gas chromatography–mass spectrometry (GC-MS) according to the method of Kaiser and Tollsten (1995) on Carbowax GC-MS columns.

In subsequent analyses, the scent of six short- and 12 long-spurred inflorescences from different sites was concentrated using polyacetate bags and trapped over approx. 30 min on filters filled with 1 mg of tenax<sup>®</sup> and 1 mg of carbotrap<sup>®</sup> activated charcoal. The samples were thermally desorbed and analysed on a Varian CP-3800 gas chromatograph (Varian, Palo Alto, CA, USA) with a 30 m  $\times$  0.25 mm internal diameter (film thickness 0.25  $\mu\text{m}$ ) Varian VF-5ms (DB5 equivalent) column coupled to a Varian 1200 quadrupole mass spectrometer.

The relative amount of each compound was expressed as a percentage of the total ion count for all compounds not also present in control samples. A similarity matrix of these square-root transformed values was calculated using the Bray–Curtis method and non-metric multidimensional scaling (NMDS) used to visualize the data using Primer 6.1.6 (Clarke and Gorley, 2006). These data were compared using two-way ANOSIM in Primer 6.1.6 with morph and column type treated as explanatory factors.

To locate the site of scent emission, freshly harvested flowers were immersed in neutral red dye (Dafni *et al.*, 2005). Because both forms of *E. parviflora* are deceptive and no nectar was found in any flowers, stained parts of the flower are likely to indicate the position of osmophores, rather than nectaries.

#### Scent choice experiments

To determine the response of pollinators to the scent of the two forms, we 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 arm was 150 mm long with a common base arm of 150 mm. The clear perspex tubing was 60 mm in diameter (Supplementary Data Fig. S1). Pollinators were introduced to the olfactometer and their choices recorded. Although this experiment was attempted using bees and beetles, bees did not move within the olfactometer. Insects for these experiments were collected from sites where the

plants were not present to avoid prior conditioning, but were within the general range of the plants. Insects were kept without food in a cool dark cage for 24 h before the experiment. Experimental trials 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 to diffuse light. The alignment of the olfactometer in relation to the sun was critical even within the greenhouse. When the axis of the olfactometer was aligned directly at the sun, the beetles moved randomly through the olfactometer (of 43 beetles, 21 selected one arm and 22 the other arm;  $\chi^2 = 0.02$ ,  $P = 0.879$ ; Fig. 7A).

Once the olfactometer was correctly calibrated with beetles 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), flow rates were re-balanced, beetles were introduced and their choices were recorded. We tested two combinations of scents: the scent of the short-spurred form of the orchid against a blank control with no odour; and the scent of the short-spurred flowers against the scent of the long-spurred form. Beetle choices between presented scents or between scent and a blank control were compared using a  $\chi^2$  goodness of fit test.

#### Visitation rates and pollen transfer efficiency

Between 28 and 268 flowers from between four and 98 inflorescences (Supplementary Data Table S5) from different populations were sampled and scored for pollinaria removal and pollinia deposition. We also determined the number of flowers that showed any sign of visitation as well as flowers that had their anther caps disturbed, which represents a failed visit. Finally, we calculated the pollination transfer efficiency as the percentage of removed pollinia (removed pollinaria multiplied by two) that are deposited on the stigmas (Johnson *et al.*, 2005a). The averages for each population, pooled where necessary across years, were compared using Mann–Whitney U-test.

#### Translocation experiment

A translocation experiment was conducted to test the prediction that the pollination success of an ecotype would be higher in its native range than in the range of the other ecotype. This experiment was only possible for the long-spurred form which is restricted to the coastal zone (the short-spurred form has a wide natural distribution; Fig. 2).

A large population of the long-spurred form flowered in a fire-break shortly before this area was scheduled to be burnt. We therefore harvested these inflorescences before they were burnt without disturbing the below-ground tuber stock and used them for a translocation experiment. Inflorescences were inspected, and flowers showing signs of either pollinarium removal or pollinia deposition 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 km outside the natural distribution of the long-spurred form, but in amongst a population of the short-spurred form. Inflorescences were left for 7 d before the flowers were

inspected for signs of pollen removal and deposition. Data were compared between sites using a generalized linear model (GLM) with a binomial error distribution and a logit link function, implemented in SPSS 19 (IBM Corp.). Significance was assessed using likelihood ratios.

#### Spur-shortening experiment

To investigate the role of floral morphology in pollinator effectiveness, we shortened the length of the spurs of the long-spurred form at the Stockville Valley site by approx. 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 clamp was used to hold the spur closed. For pairs of adjacent plants, we assigned one plant to the shortening treatment and left the other unmanipulated to serve as a control. Flowers on both treatment and control plants were first examined for any signs of visitation, and visited flowers were removed before the treatment was performed. Flowers were left for 5 d and then inspected for pollen removal or deposition. The proportion of flowers with pollinia removed was compared between treatments using a GLM with a binomial error distribution and a logit link function.

#### Self-compatibility and pollinator dependence

To test the degree of self-compatibility and potential for autonomous self-pollination which is common in *Eulophia* (Williamson, 1984; Peter and Johnson, 2009a), inflorescences of both morphs were bagged to exclude pollinators and then flowers were self-pollinated, cross-pollinated or left unmanipulated. Breeding system experiments were conducted at the Victoria Country Club and Stockville Valley sites on the short- and long-spurred forms, respectively. In addition, reciprocal crosses were made between the two forms to establish their genetic compatibility. Following the treatments, bagged flowers were allowed to develop to mature capsules and harvested shortly before dehiscence. Capsules and their seed contents were weighed and then

the seeds from a single capsule were homogenized and four random samples of 50 seeds were scored for the presence or absence of embryos. Combined capsule and seed weights and the percentage of fertile seeds produced were compared using a Kruskal–Wallis test as the data did not conform to known distributions.

## RESULTS

#### Morphometric analysis

Multivariate analysis of floral and vegetative characters revealed two distinct clusters in the phenotype space, associated with plants that possess either long or short spurs (Fig. 3). Most characters measured differed significantly between the two forms (Supplementary Data Table S2). Spur length shows a clear bimodal distribution with no overlap between the two forms (Fig. 4; Supplementary Data Table S2).

#### Colour

The colours of the two forms are nearly identical and overlap extensively in both analyses (Supplementary Data 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 Data 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 a zone of overlap of the two forms near Durban, where the short-spurred form is found at lower altitude (Fig. 2, site 1) and in the north-east part of the range where the long-spurred form

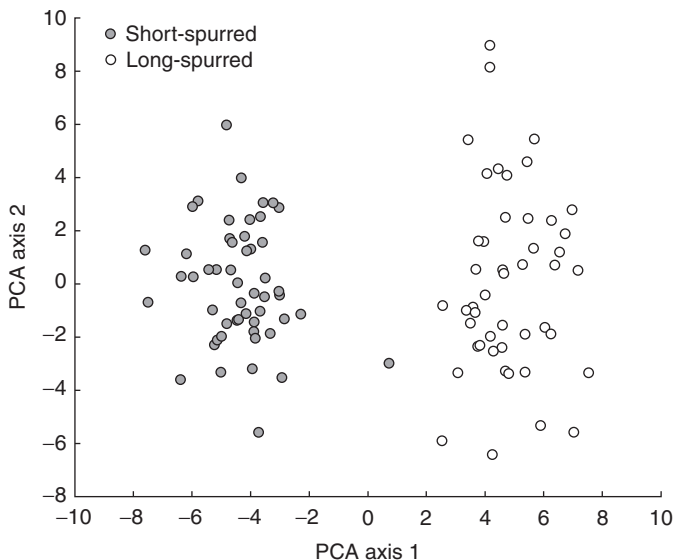


FIG. 3. Principle component analysis of the morphological characters of the long- ( $n = 47$ ) and short- ( $n = 52$ ) spurred forms of *E. parviflora*.

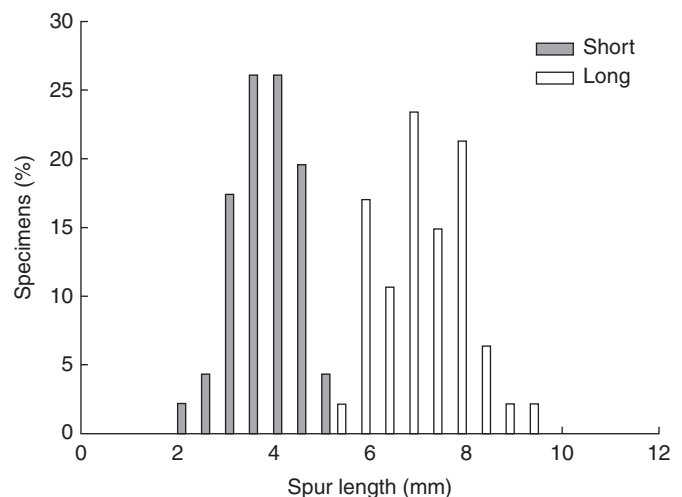


FIG. 4. The bimodal distribution of spur length in *Eulophia parviflora* corresponding to the long- ( $n = 47$ ) and short-spurred ( $n = 52$ ) forms.

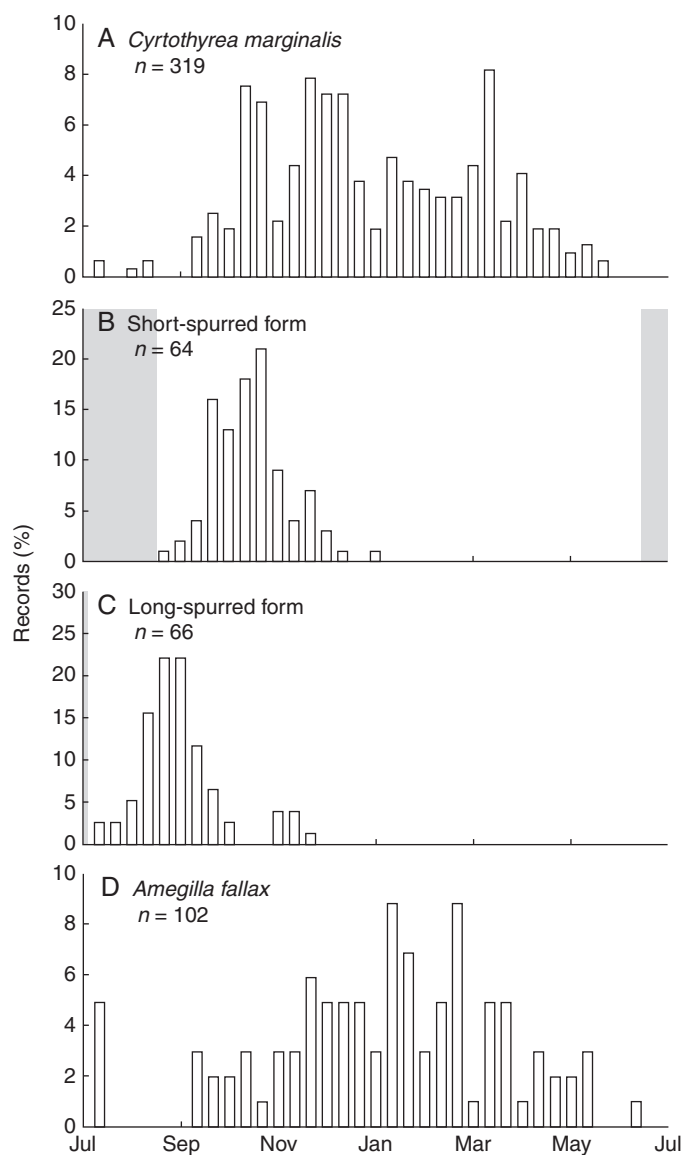


FIG. 5. Flowering phenology of the short- (B) and long-spurred (C) forms of *Eulophia parviflora* in relation to the flight periods of *C. marginalis* (A) and *A. fallax* (D), their respective pollinators. The average frost periods for sites where each form is found is indicated in grey.

which otherwise has a coastal distribution is 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 in response to late fires.

In both forms, flowering of the orchids corresponds to the emergence of their respective pollinators (described below), although the flight period of the pollinators extends much later than flowering of the orchids (Fig. 5A, D). While there are a few unusual flowering records, flowering of both forms typically commences 30 d after the last day of frost in their respective distributions (Fig. 5B, 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 occupied by the short-spurred form, extending from the middle of June to early August.

#### Insect pollinators

We collected or inspected a total of 589 *Cyrtothyrea marginalis* (Cetoniinae; Scarabaeidae) beetles visiting either the short-spurred form of *E. parviflora* or other species (primarily Asteraceae, but also Iridaceae and Hyacinthaceae) in the vicinity of the orchids. A total of 61 of the beetles of both sexes that we observed or collected at six sites bore pollinaria or viscidia of this form of the orchid (Fig. 2; Supplementary Data Table S3). The short open spur of this form accommodates the blunt anterior of the beetles (Figs. 1B, C).

In contrast, the long-spurred form appears to be pollinated by the solitary bee *Amegilla fallax* (Apidae; Anthophorinae) at the sites we examined. We collected 81 of these bees of both sexes at two sites near Durban (Fig. 2; Supplementary Data Table S3). Of these, 25 (to include most recent observations as listed in the revised supplementary table S3) bees bore pollinaria or viscidia of the long-spurred form. The majority of bees were collected while foraging on co-occurring nectar plants, but we did observe two direct visits to inflorescences. These bees have relatively long proboscides which are matched by the long, slender spurs of the long-spurred form (Fig. 1E, F).

#### Visit times and pollinarium reconfiguration

We timed five visits of *C. marginalis* beetles to inflorescences of the short-spurred form. During these visits, beetles alighted and usually clambered around the inflorescence, entering two or three flowers and depositing or extracting pollinia (Supplementary Data Video). Visits by the beetles to the inflorescences lasted 69 s on average ( $n = 5$ , range: 20–110 s). This is shorter than the average pollinarium reconfiguration time of 119 s (Supplementary Data Fig. S3).

Most of the fast moving *A. fallax* bees were collected while foraging for nectar on a variety of other food plants. We did, however, observe and record the duration of two visits (of 25 and 22 s) to inflorescences of the long-spurred form. As in the case of the short-spurred form, visit times by *Amegilla* bees to the long-spurred inflorescences (mean 23.5 s) were shorter than the mean pollinarium reconfiguration time of 28 s (Supplementary Data Fig. S3). The pollinarium bending time of the long-spurred form is significantly shorter than that of the short-spurred form ( $U = 0$ ,  $z = -9.53$ ,  $P < 0.0001$ ).

#### Scent analysis

Non-metric multidimensional scaling analysis revealed that there are discrete clusters in scent phenotype space that correspond to each morph (Fig. 6). Although the phase of the GC column used in the analyses has a slight effect on the position of these clusters in the scent phenotype space (Fig. 6), there were highly significant differences in scent composition between the two morphs that were independent of column phase (two-way ANOSIM:  $R = 0.956$ ,  $P < 0.0001$ ).

The scent of the short-spurred form is dominated by aromatic benzenoid compounds such as benzaldehyde, 4-methoxybenzaldehyde, benzyl alcohol, benzyl benzoate and methyl



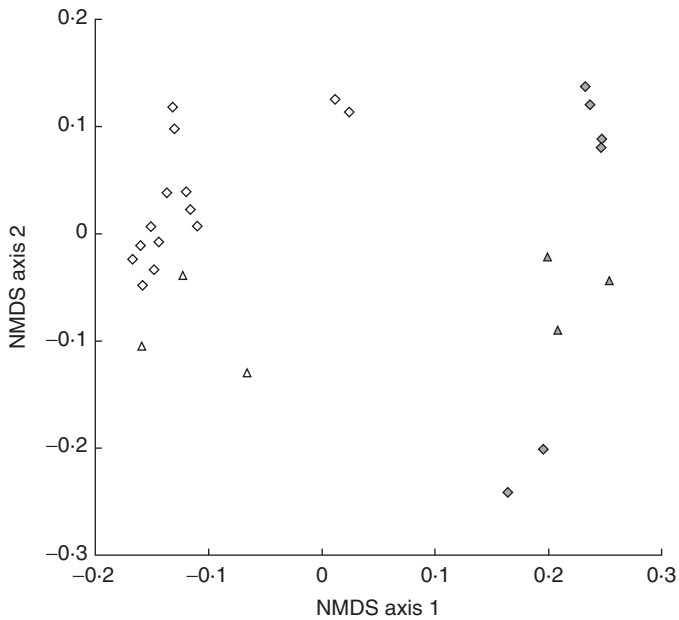


FIG. 6. Non-metric multidimensional scaling representation of the scent composition of the long-spurred (open symbols) and short-spurred (solid symbols) forms of *Eulophia parviflora* as determined using GC-MS with different columns: Carbowax (triangles) and DB5 (diamonds). The scent chemistry of the two forms is significantly different (ANOSIM,  $R = 0.956$ ,  $P < 0.0001$ ).

benzoate, as well as the monoterpenes linalool, trans-geraniol and geraniol (Supplementary Data 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, farnesol and 2,3-dihydrofarnesol, and the irregular terpene 6-methyl-5-hepten-2-one (Supplementary Data Table S4).

Only the central, rugose ridges of the central lobe of the labelum were stained by neutral red (excluding the stigmatic cavities and pollinia), indicating this as the main site of scent production in these deceptive orchids.

#### Scent choice experiments

Scent preference experiments with *C. marginalis* beetles in the Y-olfactometer revealed that these insects responded positively to the scent of flowers of the short-spurred form. Significantly more beetles (37 of 45) chose the arm of the olfactometer containing the flowers of the short-spurred form over the arm without any scent ( $\chi^2 = 18.69$ ,  $P < 0.0001$ ; Fig. 7B). The beetles showed a preference for the scent of the short-spurred form over that of the long-spurred form, with significantly more beetles (56 of 81) choosing the arm with flowers of the short-spurred form over the arm containing the scent of the long-spurred form ( $\chi^2 = 11.86$ ,  $P = 0.0005$ ; Fig. 7C).

#### Visitation rates and pollen transfer efficiency

Rates of pollinaria removal and deposition as well as overall visitation rates are significantly higher in the beetle-pollinated short-spurred form compared with the bee-pollinated long-spurred form (Supplementary Data Table S5). This translates into very high pollen transfer efficiencies in the short-spurred

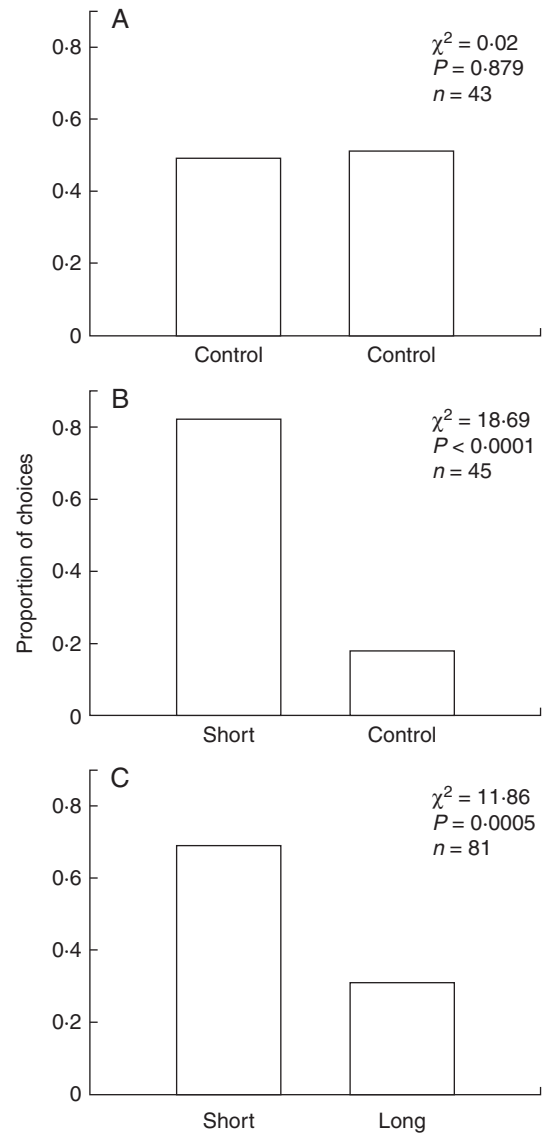


FIG. 7. Choices made by *C. marginalis* beetles in a Y-maze olfactometer. (A) Olfactometer with empty arms aligned correctly to the sun. (B) Olfactometer with one arm containing flowers of the short-spurred form of *Eulophia parviflora*. (C) Olfactometer with one arm containing flowers of the short-spurred form of *Eulophia parviflora* and the other containing flowers of the long-spurred form.

form, with nearly 25 % of all removed pollinia being subsequently deposited on stigmas. In contrast, only 6 % of removed pollinia were deposited on stigmas of the long-spurred form. Pollination failure (visits that remove the anther cap of the flower, but not the pollinarium) was slightly higher in the beetle-pollinated form.

#### Translocation experiment

There were no significant differences in mean ( $\pm$  s.e.) rates of pollinaria removal (translocated within the natural range,  $0.14 \pm 0.04$ ; translocated out of the natural range,  $0.19 \pm 0.04$ ;  $\chi^2 = 0.886$ ,  $P = 0.346$ ) and pollinia deposition (translocated within the natural range,  $0.01 \pm 0.01$ ; translocated out of the natural range,  $0.02 \pm 0.01$ ;  $\chi^2 = 0.276$ ,  $P = 0.599$ ) for 21



TABLE 1. Results of experiments to determine the breeding systems of the two forms of *Eulophia parviflora*

Form	Measurement of fecundity	Treatment					Test statistic
		Bagged and unmanipulated	Self-pollinated	Cross-pollinated	Intermorph cross		
Short-spurred	Percentage fruit set ( <i>n</i> )	0 ( <i>n</i> = 18)	66 ( <i>n</i> = 29)	70 ( <i>n</i> = 26)	85 ( <i>n</i> = 7)		
	Mean ( $\pm$ s.e.) capsule and seed mass in g* ( <i>n</i> )	—	0.48 $\pm$ 0.08 ( <i>n</i> = 19)	0.56 $\pm$ 0.09 ( <i>n</i> = 18)	0.33 $\pm$ 0.05 ( <i>n</i> = 6)	$H = 1.731, P = 0.421$	
	Mean ( $\pm$ s.e.) percentage of seeds with embryos* ( <i>n</i> )	—	44.7 $\pm$ 6.5 <sup>a</sup> ( <i>n</i> = 19)	83.8 $\pm$ 4.1 <sup>b</sup> ( <i>n</i> = 18)	86.3 $\pm$ 2.5 <sup>b</sup> ( <i>n</i> = 6)	$H = 19.88, P < 0.0001$	
Long-spurred	Percentage fruit set ( <i>n</i> )	0 ( <i>n</i> = 32)	Not recorded	Not recorded	Not recorded		
	Mean ( $\pm$ s.e.) capsule and seed mass in grams* ( <i>n</i> )	—	0.22 $\pm$ 0.02 ( <i>n</i> = 9)	0.23 $\pm$ 0.03 ( <i>n</i> = 8)	0.29 $\pm$ 0.05 ( <i>n</i> = 7)	$H = 1.70, P = 0.428$	
	Mean ( $\pm$ s.e.) percentage of seeds with embryos* ( <i>n</i> )	—	20.2 $\pm$ 5.3 <sup>a</sup> ( <i>n</i> = 9)	86.6 $\pm$ 1.6 <sup>b</sup> ( <i>n</i> = 8)	84.5 $\pm$ 1.6 <sup>b</sup> ( <i>n</i> = 7)	$H = 16.31, P = 0.0003$	

\*Mass and seed set data were compared using Kruskal–Wallis test. Homogenous groups were identified using Mann–Whitney post-hoc pairwise test and indicated with letters.

inflorescences of the long-spurred form translocated within a natural population and 16 inflorescences translocated 60 km outside the natural distribution range.

#### Spur shortening experiment

Experimental shortening of the spurs of the long-spurred form significantly reduced the mean ( $\pm$  s.e.) proportion of flowers with pollinaria removed on inflorescences from  $0.35 \pm 0.054$  to  $0.06 \pm 0.027$  ( $\chi^2 = 21.926, P < 0.0001$ ). Pollen deposition on flowers in both treatment groups was too infrequent for statistical analysis.

#### Breeding system

Both forms of *E. parviflora* showed evidence of self-compatibility, with capsules produced by the self-treatment comparable in weight with those produced by cross-pollination. In both forms, however, the quality of seed produced in the self-pollinated capsules was significantly lower than that produced by outcrossing (Table 1). In the short-spurred plants, selfed capsules produce 50 % fewer fertile seeds (as indicated by the presence of an embryo) while in the long-spurred form, selfed capsules produced 75 % fewer fertile seed (Table 1). Hand-pollinated flowers usually resulted in fruit production, but none of the bagged and unmanipulated flowers, in both forms, set fruit, indicating that the plants are not autogamous. The two forms appear to be interfertile, with reciprocal crosses leading to fruits and seeds of quality comparable with those produced by cross-pollination within each form (Table 1).

## DISCUSSION

The results of this study are consistent with the specific predictions generated by the hypothesis that floral divergence between the two forms of *Eulophia parviflora* reflects an evolutionary shift between different pollinators. Specifically, these analyses show that there are at least two forms in the complex, differing in flower shape (Fig. 3), scent (Supplementary Data Table S4), phenology (Fig. 5) and pollinarium reconfiguration time (Supplementary Data Fig. S3). Our observations at a wide range of sites suggest 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. Coexistence of the two forms at some sites near Durban substitutes 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.

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 as spur length (Robertson and Wyatt, 1990; Johnson, 1997; Johnson and Steiner, 1997; Boyd, 2004), colour (Newman *et al.*, 2012), scent chemistry (Pellmyr, 1986; Johnson *et al.*, 2005b) and flowering phenology (Herrera *et al.*, 2002), although see studies in this special issue (e.g. Sun *et al.*, 2014; van der Niet *et al.*, 2014). Interestingly, we found no quantitative difference between the flower colours

of the two forms of *E. parviflora* (Supplementary Data Fig. S2), which suggests this trait was not important for mediating the transition between bee and beetle pollination systems.

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 and Edwards, 1989; Cherry and 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 and Petroski, 2004; Steenhuisen *et al.*, 2013), benzyl benzoate (Leal *et al.*, 1994) and linalool (Donaldson *et al.*, 1990; Imai *et al.*, 1998). Further experiments are required to unravel the relative attractiveness of the different compounds making up the scent of the short-spurred form.

There are relatively few reports of beetle pollination in orchids, and in most of these cases the flowers are reported to be scented and pale green or brown in colour (Nilsson, 1981; Singer and Cocucci, 1997; Peter and Johnson, 2006a; Johnson *et al.*, 2007; Pedersen *et al.*, 2013). The pollination system of the short-spurred form of *E. parviflora* has similarities to that of *Eulophia* (*Pteroglossaspis*) *ruwenzoriensis* which is also pollinated by cetoniid beetles and has dense inflorescences of scented flowers and petals with dark adaxial surfaces (Singer and Cocucci, 1997). However, *E. ruwenzoriensis* has drab pale pink and green inflorescences; is rewarding, producing 'jelly-like' nectar; and has a yeast-like scent. The short-spurred beetle-pollinated form is also similar to *Ceratandra grandiflora*, a yellow-flowered orchid with dense inflorescences. Monkey beetles aggregate on the deceptive inflorescences of *C. grandiflora* which serve as mating rendezvous sites (Steiner, 1998). Crowded inflorescences (Character 14, Supplementary Data Table S1) appear to be an important characteristic of many beetle-pollinated orchids (Singer and Cocucci, 1997; Steiner, 1998; Peter and Johnson, 2006a, 2009b) and are consistent with the general syndrome of beetle pollination (van der Pijl, 1961; Faegri and van der Pijl, 1966; Bernhardt, 2000), but see Pedersen *et al.* (2013). Crowded inflorescences appear to be an efficient way of producing large attractive displays from small flowers, and also allow beetles to clamber from flower to flower without flying (Supplementary Data Video).

The only bee species found to carry pollinaria of this morphotype at the sites we examined was *A. fallax* (both sexes), but we cannot exclude the possibility that other medium- to small-sized solitary bees contribute to pollination. The two bees observed visiting flowers exhibited a typical zig-zag, scent-orientating 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 sesquiterpene farnesene (Supplementary Data Table S4), compounds known 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 and Bergström, 1997). *Xylocopa* bees may have a 'dispersed lek system', with males competing over large areas by scent-marking territories to attract female bees for mating (Andersen *et al.*, 1988; Minckley *et al.*, 1991). Male *Bombus* bees have a similar scent mark behaviour to attract virgin queens (Bergman and Bergström, 1997). It is possible, therefore, that farnesene derivatives play a role in the biology of *A. fallax*, either to mark communal roost positions or to co-ordinate the mating rendezvous. If this is the case, this may represent a novel aspect of pollinator deception that has not been documented previously. Johnson *et al.* (2005b) speculated that the attraction of both male and female *Tetraloniella* (Anthophorinae) bees, known to have a strong lekking behaviour (C. 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.

Long-spurred forms in the *E. parviflora* complex are found mainly at low altitude along the coast, while the short-spurred form is found mainly at high altitudes (Fig. 2). The ultimate basis for the evolution of these geographical forms within the *E. parviflora* complex remains uncertain. As both pollinator species have wide distributions in southern Africa (Holm and Marais, 1992; Eardley, 1994), there is not the underlying geographical gradient in pollinator availability that would be expected from the Grant–Stebbins model. The wide distribution of the pollinators probably explains why the prediction that forms would have higher pollination success in their own distribution range was not upheld by the translocation experiment.

A possible alternative explanation for the distribution of the two forms is suggested by comparison of flowering and phenology times of respective forms and their pollinators as well as local frost periods (Fig. 5). 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 *A. fallax* bees. This early flowering coinciding with newly emerged bees is possible at the coast because of the lack of frost, but may not be viable at colder inland localities.

A recent species-level phylogeny for *Eulophia* (Martos *et al.*, 2014) suggests that beetle pollination is derived in the short-spurred form in an otherwise bee-pollinated clade characterized by lax inflorescences with few resupinate flowers open at one time (Peter and Johnson, 2008, 2009c, 69). This apparent shift from bee to beetle pollination is associated with the evolution of shorter spurs and is at odds with the general evolutionary trend for shifts from shorter to longer floral tubes (Whittall and Hodges, 2007). It is noteworthy that pollination success of the beetle-pollinated form is almost always higher than that of the longer-spurred bee-pollinated form.

Finally, our investigations of the *E. parviflora* complex suggest that the short- and long-spurred forms could be considered closely related sister species rather than intraspecific variants. Although the crossing experiments suggest that the two forms are interfertile (at least capable of forming hybrid seeds with embryos), the two forms 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 and Orr, 2004) leads us to conclude that the forms arose as ecotypes and have reached the stage of being 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.

#### SUPPLEMENTARY DATA

Supplementary data are available online at [www.aob.oxford-journals.org](http://www.aob.oxford-journals.org) and consist of the following. Table S1: mean character states for the two forms. Table S2: Eigenvalues, percentage variance explained and cumulative variance explained for the PCA analysis. Table S3: pollinators collected visiting or bearing pollinaria of the two forms of *E. parviflora*. Table S4: floral scent composition for plants of each of the two forms of *Eulophia parviflora* analysed using GC-MS fitted with either a Carbowax or a DB5 column. Table S5: pollen removal, deposition and pollen transfer efficiencies in different populations of the two forms of *E. parviflora*. Figure S1: diagram of the Perspex Y-shaped olfactometer used for determining beetle scent preferences. Figure S2: comparison of flower colours of short- and long-spurred forms summarized using the Endler segment classification method and Chittka bee colour hexagon. Figure S3: mean pollinator reconfiguration and mean pollinator visit time to inflorescences of the two forms. Video: short film showing a visit by *Cyrtothyrea marginalis* to the short-spurred form of *E. parviflora*.

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