New Phytologist

Disentangling visual and olfactory signals in mushroom-mimicking *Dracula* orchids using realistic three-dimensional printed flowers

Tobias Policha[1], Aleah Davis[1], Melinda Barnadas[2,3], Bryn T. M. Dentinger[4,5], Robert A. Raguso[6] and Bitty A. Roy[1]

[1]Institute of Ecology & Evolution, 5289 University of Oregon, Eugene, OR 97403, USA; [2]Department of Visual Arts, University of California, San Diego, 9500 Gilman Dr., La Jolla, CA 92093, USA; [3]Magpie Studio: Fabrication for Art and Science, La Jolla, CA 92092, USA; [4]Jodrell Laboratory, Royal Botanic Gardens, Kew, Richmond, Surrey, TW9 3DS, UK; [5]Institute of Biological, Environmental and Rural Sciences, Aberystwyth University, Cledwyn Building, Penglais, Aberystwyth, Ceredigion, SY23 3DD, UK; [6]Department of Neurobiology and Behavior, Cornell University, Ithaca, NY 14853, USA

Author for correspondence:
Tobias Policha
Tel: +1 541 513 8263
Email: tpolicha@uoregon.edu

Received: *1 September 2015*
Accepted: *1 December 2015*

New Phytologist (2016) **210:** 1058–1071
doi: 10.1111/nph.13855

Key words: cloud forest, *Dracula lafleurii*, Drosophilidae, GC-MS, mimicry, neotropical, Orchidaceae, pollination.

Summary

- Flowers use olfactory and visual signals to communicate with pollinators. Disentangling the relative contributions and potential synergies between signals remains a challenge. Understanding the perceptual biases exploited by floral mimicry illuminates the evolution of these signals. Here, we disentangle the olfactory and visual components of *Dracula lafleurii*, which mimics mushrooms in size, shape, color and scent, and is pollinated by mushroom-associated flies.
- To decouple signals, we used three-dimensional printing to produce realistic artificial flower molds that were color matched and cast using scent-free surgical silicone, to which we could add scent. We used GC-MS to measure scents in co-occurring mushrooms, and related orchids, and used these scents in field experiments.
- By combining silicone flower parts with real floral organs, we created chimeras that identified the mushroom-like labellum as a source of volatile attraction. In addition, we showed remarkable overlap in the volatile chemistry between *D. lafleurii* and co-occurring mushrooms.
- The characters defining the genus *Dracula* – a mushroom-like, 'gilled' labellum and a showy, patterned calyx – enhance pollinator attraction by exploiting the visual and chemosensory perceptual biases of drosophilid flies. Our techniques for the manipulation of complex traits in a nonmodel system not conducive to gene silencing or selective breeding are useful for other systems.

Introduction

Floral mimicry and deceptive pollination have been recognized for over 200 yr, with Sprengel (1793) referring to 'false nectar flowers' in his seminal 'The secret of nature in the form and fertilisation of flowers discovered', but the details of the perceptual biases involved in the evolution of floral mimicry remain an active field of investigation (Dafni, 1984; Schiestl, 2005; Jersáková *et al.*, 2006; Vereecken & McNeil, 2010). Our understanding of the evolution of mimicry in general is largely based on concepts developed to explain predator avoidance in animals (Roy & Widmer, 1999). Floral mimicry differs because flowers function to attract pollinators, rather than avoid them. Floral mimicry in plants thus evolves through the selection of floral traits by pollinators. In fact, phylogenetic evidence suggests that the enormous diversity of orchids may be a consequence of the early emergence and ubiquity of deceptive pollination strategies (Cozzolino & Widmer, 2005).

Flowers evolve in response to the perceptual biases of pollinators for traits such as scent, color and pattern (Schaefer & Ruxton, 2009; Schiestl & Dötterl, 2012; Papadopulos *et al.*, 2013). Small differences in perceptual acuity and bias can be exploited by selection if they impart an increase in fitness (Chittka *et al.*, 2001). Perceptual biases do not have to be innate; they can also be learned preferences, or can emerge from a combination of causes (Schaefer & Ruxton, 2009). From the floral perspective, it does not matter which mechanism imparts the bias; natural selection on the flowers will favor individuals that match the perceptual biases of their most effective pollinators most closely, if these increase the fitness of the plants.

Legitimate fly pollination (myophily) is widespread in orchids and other flowering plants (Johnson & Midgley, 1997; Larson *et al.*, 2001; Ollerton *et al.*, 2009), and flies are also exploited in various ways to act as pollinators in deceptive scenarios, including brood site mimicry (Burgess *et al.*, 2004; Van der Niet *et al.*, 2011; Jürgens *et al.*, 2013), yeast mimicry (Goodrich *et al.*, 2006;

Stokl *et al.*, 2010) and sexual deception (Johnson & Midgley, 1997; Blanco & Barboza, 2005; Ellis & Johnson, 2010; Gaskett, 2011). As a result of their plenitude, flies represent an important potential source of pollination services for plants (Larson *et al.*, 2001; Ssymank *et al.*, 2008). In moist, shady habitats, such as tropical cloud forests, which are typically poor in anthophilous insect fauna, but rich in mushroom-associated taxa, the evolution of pollination by these mushroom-associated flies should be favored (Mesler *et al.*, 1980), yet the species-rich genus *Dracula* is one of only a few genera suspected to mimic mushrooms (Vogel, 1978; Jersáková *et al.*, 2006).

Dracula orchids are curious because their labella look like co-occurring mushrooms (Fig. 1) (see Supporting Information Fig. S1 for images of co-occurring mushrooms) (Vogel, 1978; Dentinger & Roy, 2010). Although previous studies have shown that some *Dracula* species produce the same volatile compounds that give mushrooms their characteristic odors (Kaiser, 1993, 2006; Policha, 2014), and that they are pollinated by mushroom-visiting drosophilid flies (Fig. 1) (Endara *et al.*, 2010; Policha, 2014), the relative importance of visual and olfactory floral traits to pollinator attraction remain unknown in this system. What are the implications for flower signaling of having mushroom flies as pollinators? The flying insect community at our field site in Ecuador is dominated by dipteran groups known to include mushroom-visiting members (Phoridae, Sciaridae, Mycetophilidae), and the flies that we find at *Dracula* flowers come from largely mycophilous genera of the Drosophilidae (Endara *et al.*, 2010; Policha, 2014). Mushroom-visiting flies are often polyphagous as a consequence of their dependence on an ephemeral resource (Jaenike, 1978). As generalists, these insects may use imprecise search images that are particularly susceptible to exploitation by mimicry. Models of associative learning in a mimetic context suggest that learned avoidance should be impaired when fraudulent signals are more variable (Balogh *et al.*, 2008). Our data on the mushrooms that co-occur with *Dracula* orchids indicate that there is no single mushroom model (Dentinger & Roy, 2010). Instead, *Dracula* orchids appear to represent a generalized mimicry system, in which there is a convergence on the mean phenotype of co-occurring mushrooms (B. T. M. Dentinger *et al.*, unpublished). In this situation, *Dracula* flowers are likely to benefit from the exploitation of perceptual biases in the visitors (Ruxton & Schaefer, 2011).

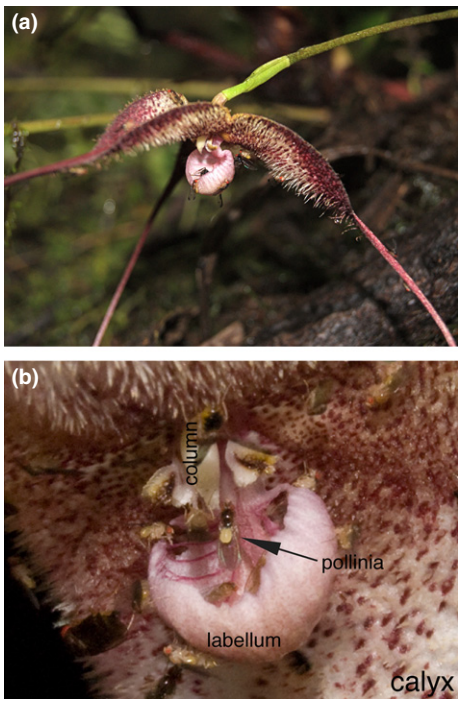

Fig. 1 Flowers of *Dracula lafleurii* from our research site (the type locality), Reserva Los Cedros, Ecuador. (a) *Dracula lafleurii in situ* with drosophilid visitors. The descending, few-flowered racemes produce pendant, umbrella-like flowers with subglobose mushroom-like labella. © B. A. Roy. (b) Intrafloral morphology: calyx, column, labellum with radiating 'gill-like' ridges (*c.* 1 cm across for scale) and floral visitors in the genus *Zygothrica* (Drosophilidae), one with a pollinium attached to its thorax. © B. T. M. Dentinger.

We examine the signaling motifs enabling this mimicry using *Dracula lafleurii*, which has relatively large flowers that it presents over several months (Fig. 1). Is the mimicry multimodal, utilizing visual and olfactory signals? Or is scent the primary attractant, as is typical in carrion brood-site mimicry systems (Stensmyr *et al.*, 2002; Moré *et al.*, 2013), with visual or other signals contributing secondarily? In addition to the mushroom-like labellum, *D. lafleurii* flowers display rather large (area = 913.5 mm^2, SE = 68; n = 22 flowers) and showy, fused sepals (Fig. 1), suggesting that there may be a significant visual component independent of the mushroom motif.

Artificial flowers often are used to experimentally decouple floral traits (Raguso, 2006), and recent studies have used three-dimensional (3D) printing to generate simplistic funnel- or disc-shaped floral dummies (Campos *et al.*, 2015). Here, we take the 3D printing approach further to produce realistic artificial flowers and flower parts that enable us to experimentally decouple the highly complex phenotype of *Dracula* flowers in the remote field setting of an Ecuadorean cloud forest. Our results show that both visual and olfactory components play a role in attraction in this system and suggest that each floral part makes a discernible contribution to the recruitment of dipteran pollinators. We show that the gilled labellum – the feature that defines the genus *Dracula* – is largely responsible for the effective mushroom phenotype, and we discuss possible functional roles for the showy calyx.

Materials and Methods

Study site

Los Cedros Biological Reserve, the type locality for *Dracula lafleurii* Luer & Dalström (Luer, 1993), is located between 1250 and 2200 m elevation on the western slope of the Andes of northwestern Ecuador (00°18′31.0″N, 78°46′44.6″W). This private reserve protects *c.* 7000 ha of mostly primary montane cloud forest (Sierra, 1999), abutting the 305 000 ha Cotocachi-Cayapas Ecological Reserve. It is part of the Chocó phytogeographical zone, recognized as one of the most biologically diverse habitats on earth (Myers *et al.*, 2000). Indeed, tree diversity at the reserve is high – estimated at 300 tree species ha^{-1} (Peck *et al.*, 2008).

Rainfall averages 2903 ± 186 mm yr^{-1} ($n = 15$ yr), based on records kept at the reserve (J. DeCoux, pers. comm.). Our studies were conducted during the local rainy season (January–March) 2010–2012, when *Dracula* orchids were in peak bloom. The focal populations of *D. lafleurii* were situated at *c.* 1300 m elevation next to the Rio Los Cedros.

Dracula lafleurii and visitors

The genus *Dracula* (*c.* 130 spp.; The Plant List, 2013) is part of the diverse Neotropical subtribe Pleurothallidinae (Epidendroideae), which accounts for 15–20% of the species diversity in the Orchidaceae, and which, as a group, is generally thought to be fly pollinated (Van der Pijl & Dodson, 1966; Pridgeon *et al.*, 2001; Van der Cingel, 2001). *Dracula* orchids reach their peak diversity in the western Andean cloud forests of Colombia (>60 spp.) and Ecuador (>45 spp.) (Luer, 1993; Jorgensen & León-Yanez, 1999).

This study focuses on *D. lafleurii* (Figs 1, S2), which produces pendant, umbrella-like flowers with subglobose labella on descending, successively few-flowered racemes (*c.* 1–5 racemes at a time; Fig. 1). Flowers can remain open and attractive to flies for *c.* 10 d (Endara *et al.*, 2010; T. Policha *et al.*, unpublished). The calyces range from 411 to 1623 mm^2 in area, the petals are 3×2 mm^2 in size and the labellum averages *c.* 1 cm in width. The flowers do not produce detectable levels of nectar (Endara *et al.*, 2010).

Our earlier work has shown that *D. lafleurii* is pollinated by small flies in the family Drosophilidae (Endara *et al.*, 2010; McNeil, 2013; Policha, 2014); see pollinium on fly in Fig. 1 and video from McNeil (2013). In this study, we collected *c.* 20 different species of flies from these flowers, mostly undescribed species (D. Grimaldi, pers. comm.) in the genus *Zygothrica* (Drosophilidae). At least 11 fly species have been observed removing pollinia (Policha, 2014). We have occasionally seen nonpollinating ants and staphylinid beetles in the flowers, and there are frequent visits by tiny flies in the family Phoridae. However, based on their size, it is unlikely that phorids could remove pollinia or travel with a pollinium attached to them. Indeed, the only times we have seen pollinia removed have been on the thoraxes of drosophilid flies (Fig. 1). In the following analyses, we only include Drosophilidae large enough to remove pollinia. These drosophilids display a range of behaviors on the flowers, including standing (sheltered from the rain), walking (flies are known to taste with their feet; Dethier, 1976; Barth, 1985) and lapping at the surface and apparently consuming yeasts that occur there (McAlpine, 2013). They commonly display territorial behavior, courtship-associated wing movements (Grimaldi, 1987; Burla, 1990) and mating has also (rarely) been observed (see video from McNeil (2013) of our work, for an example of visitor behavior, including two fly species with a pollinium attached).

Successful pollination rates are low in *D. lafleurii* (5.7% of buds result in fruits; Policha, 2014). Pollinia removal is often realized after an individual wedges itself deep within the flower, spending from several minutes to >1 h beneath the column (Endara *et al.*, 2010; Policha, 2014). We have also observed flies with pollinia spend up to 15 min on a flower even after pollinia removal. To reach the column, the fly must cross the adaxial portion of the labellum, making it an important target of attraction.

Artificial flowers

Melinda Barnadas manufactured the artificial flowers (see later, Figs 3, 4, 6) using scent-free, pharmaceutical-grade silicone. Molds were made by casting dissected flower parts in an aqueous impression material (potassium alginate and calcium sulfate, http://www.renewmaterials.com). These temporary molds were used to create stable positive casts in high-strength plaster (Hydrocal FGR-95 Gypsum Cement, USG Corp., Chicago, IL, USA). The plaster positives were scanned into high-resolution 3D images at the Arius 3D Imaging Center at the Canadian Museum of Nature (Gatineau, QC, USA), and digitally adjusted to specified proportions. The finalized 3D images were printed as cyanoacrylate impregnated gypsum molds on a ZCorp Spectrum 510 (The Scripps Research Institute, La Jolla, CA, USA). The final synthetic flower parts were created using a fragrance-free, platinum-cured silicone (Silibione® RTV 4420 A/B, Bluestar Silicones USA Corp., East Brunswick, NJ, USA). Pigments were encapsulated in silicone before casting to achieve the desired coloration without scents from the pigments. Flowers were collected (Andy's Orchids, Encinitas, CA, USA) and color matched using a spectrophotometer (Shimadzu UV-1650; range 190–1110 nm; deuterium and tungsten–halogen lamps).

Because UV reflectance can play an important role in insect attraction (Menzel, 1975; Peter & Johnson, 2008), we determined whether this would be an important aspect of visual signaling to include in the artificial flowers. From measurements of glasshouse-grown *D. lafleurii* flowers (Marsh Hollow Orchids, Fenwick, ON, Canada), what little UV reflectance there may be appears to come primarily from the column (Fig. S3). We assayed visits to artificial flowers with a white, a UV-reflective or a UV-fluorescent labellum. The UV-reflective labella were fabricated by dehydrating, powdering and adding Bird Vision UV Decoy Paint (Reel Wings Decoy Co. Inc., Fargo, ND, USA) to the silicone before creating the labellum. The UV-fluorescent labellum used Fluorescent Pigment White No. 56000 from Kremer Pigments Inc. (New York, NY, USA) added to the silicone as a dry pigment. Fluorescent pigments are luminescent materials that require no artificial energy to reflect colored light and to give off fluorescent light (Streitel, 2009). Neither UV reflectivity nor UV fluorescence had a significant effect on visitation (Fig. S3). We used the plain white pigments in subsequent experiments.

Experimental design, response variables and statistical analysis

Although earlier work has revealed that drosophilid fungus flies pollinate *Dracula* flowers (Endara *et al.*, 2010), the mechanisms by which they are attracted to such flowers remain unknown. We thus designed four manipulative field experiments to measure the responses of natural pollinators to decoupled visual and olfactory aspects of the complex floral display of *D. lafleurii*. These experiments involved real (natural) flowers that had been cloaked to

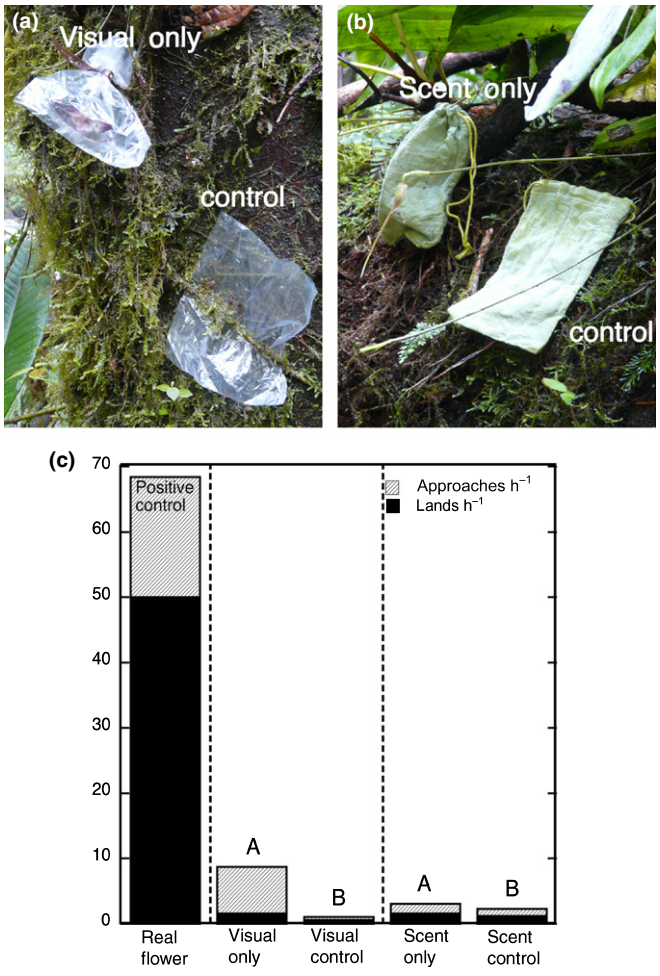

Fig. 2 Deconstructing signals in *Dracula lafleurii*. (a) A bagged 'visual only' treatment inside a scent-proof oven bag with its negative control (an empty bag). (b) A bagged 'scent only' treatment inside a green, muslin, scent-permeable bag with its negative control (an empty bag). (c) The number of approaches and lands h^{-1} as a function of treatment. Each isolated aspect of the signaling phenotype attracted more approaches than its respective controls; however, in terms of landings there were no significant differences. Treatments that are significantly different from each other are indicated by different letters; capital letters designate approaches. The dashed lines group the relevant comparisons of treatments with their respective controls. Photographs © R. Manobanda, used with permission.

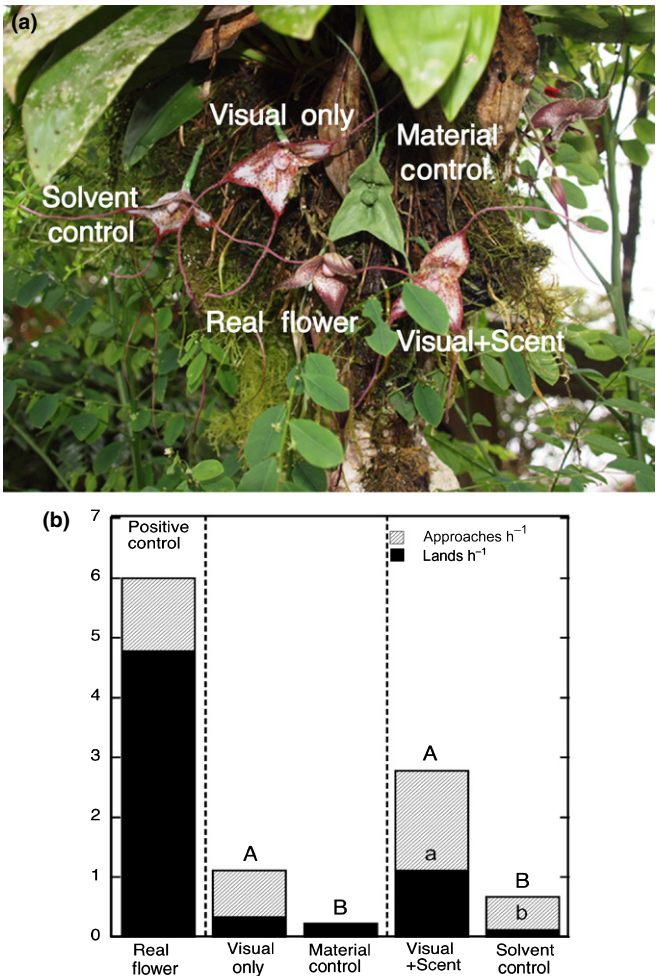

Fig. 3 (a) Treatments in the reconstruction of olfactory and visual signals of *Dracula lafleurii* using life-like artificial flowers: a living flower ('positive control', center bottom), an artificial flower ('visual only'), a green artificial flower ('material control', center, above the real flower), an artificial flower augmented with *Dracula* volatiles extracted in solvent ('visual + scent') and an artificial flower augmented with solvent only as a 'solvent control'. The added volatile extracts were prepared by soaking *D. lafleurii* flowers in a 9 : 1 hexane : acetone solvent and then diluting the supernatant with mineral oil (2 : 1). Photograph © B. A. Roy. (b) The number of approaches and lands h^{-1} as a function of treatment. Treatments that are significantly different from each other are indicated by different letters; capital letters designate approaches, small letters designate lands. There was no significant difference in the number of approaches between the real flower (positive control) and the 'visual + scent' treatment, but there was in terms of lands. The dashed lines group the relevant comparisons of treatments with their respective controls.

effectively remove their visual or olfactory presentation (Fig. 2), artificial flowers whose visual or olfactory aspects could be independently manipulated (Fig. 3, and see later Fig. 6) or chimeric flowers constructed as mosaics of real and artificial flower parts (Fig. 4). In each experiment, our field assays involved arranging a series of treatments around a blooming *D. lafleurii in situ* (Figs 3, 4). This ensured that there would be appropriate insect communities in the vicinity, allowing us to address particular hypotheses by comparing the visitation rates to particular treatments with that of a real flower as a positive control. These *in situ* plants were either found on tree trunks within reasonable reach (< 2 m high) or on downed branches. These four field experiments are summarized in Table 1 and discussed later. More details are available in Table S1.

Pollinia removal rates in orchids are not high (Bierzychudek, 1981; Smithson, 2002). However, the more flies that visit a flower, the more likely it will be that one of them will remove a pollinium. Thus, we used visitation rate as a metric of potential pollination success. In some experiments, we were also able to keep track of approaches, which were defined as directed flight to within 5 cm of the target (all approaches are included in this count, including those that resulted in a landing on the flower and those that did not), as well as visit duration. Occasionally, these additional variables were impossible to record because of

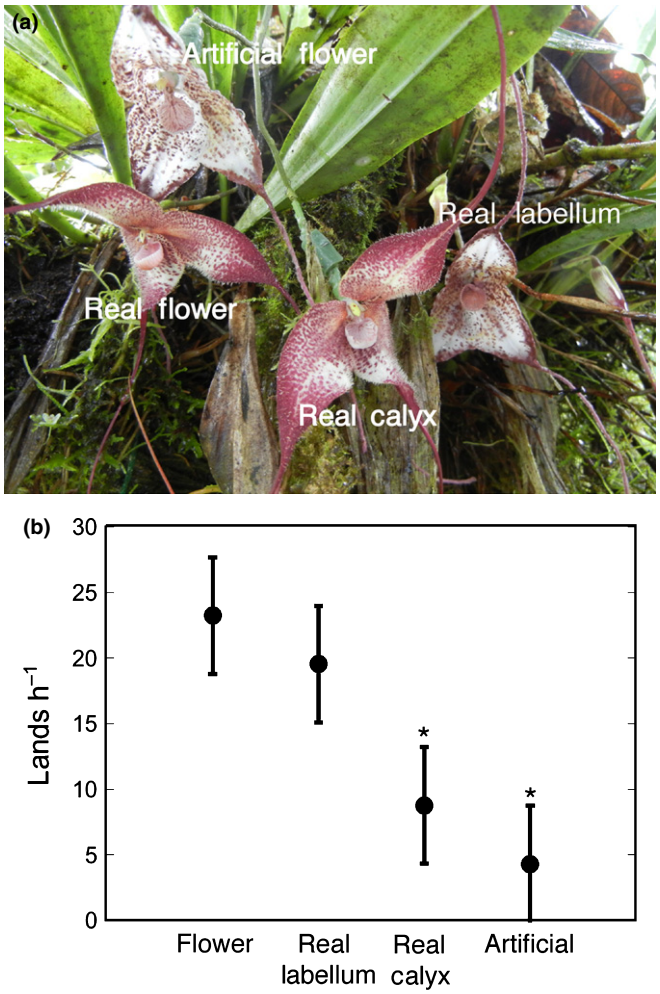

Fig. 4 Chimeric treatments, including every combination of living and artificial flower parts of *Dracula lafleurii*, indicate that the labellum is key to insect attraction. (a) The treatments. Photograph © A. Davis. (b) Number of lands h^{-1}. Asterisks indicate significant *a priori* contrasts when the treatment has fewer visits than the real flower controls (*, $P \leq 0.05$). Error bars represent ± 1SE.

the sheer volume of visitors. Generally, the results followed the same pattern across the three metrics, adding data but not new information. We have made a note of instances in which different patterns were observed.

All experiments were set up in a randomized design with observation period as the replicate. Each observation period was 30 min long. ANOVAs were performed with treatment as the main effect. ANOVAs were performed in SPSS v.22 (IBM Corp., 2013). In all cases, there was significant heterogeneity of variances among treatments according to Levene's tests. We thus used Welch's tests of equality of means for the overall model tests, and for our *a priori* contrasts we did not assume equal variances. In the assay of visual cues, one of the treatments had no visitors and thus no variance; we therefore used the nonparametric Kruskal–Wallis test for the overall model test.

Deconstruction of visual and olfactory signals To address the hypothesis that either visual or olfactory signals are sufficient to attract floral visitors, we selectively isolated each aspect of the phenotype. Sites ($n = 6$) with at least two open and attractive flowers were subjected to the following treatments (Fig. 2): an unmanipulated living flower ('real flower'); a living flower enclosed within a transparent, odor-impermeable, nylon-resin oven bag (Reynolds®, Lake Forest, IL, USA) ('visual only'); an empty oven bag ('visual control'); a living flower masked within a green muslin bag ('scent only'); an empty green muslin bag ('scent control'). Masked treatments ('scent only' or 'visual only') were observed successively next to an unmanipulated flower and the respective negative control, in random order, for 10 replicates (5 h in total). We measured both landing rates and approaches. All observations were made between 31 January and 10 February 2010.

Reconstruction of visual and olfactory signals Artificial flowers were presented in arrays of five treatments (Fig. 3; Table 1): a living flower ('positive control'); an artificial flower ('visual only'); a green artificial flower ('material control'); an artificial flower augmented with a volatile solvent extract ('visual + scent'); an artificial flower augmented with solvent only ('solvent control'). The added volatile extracts were prepared from wild *D. lafleurii* flowers at anthesis. The floral organs were partitioned by removing the calyx from the rest of the flower (corolla, including labellum/column) with a razor blade and separately soaking the calyx and the corolla/column of *D. lafleurii* flowers in 0.5 ml of a 9 : 1 hexane : acetone solvent for 6 h and diluting the supernatant with mineral oil (2 : 1). Volatile extract from the calyx was applied to the calyx of the artificial flower and the extract from the corolla/column of the flowers was applied to the labellum of the artificial flower. The unscented 'visual only' artificial flower was compared with the 'material control' to establish the effect of visual signals alone. The 'visual + scent' flower was compared with the 'solvent control' to assess the effect of the *Dracula* volatiles independent of the solvent background. The 'visual + scent' treatment was compared with the real flowers to see how completely we reconstructed floral attraction. All observations were made between 15 January and 7 March 2011, over 18 replicates (9 h in total), and we measured approaches and landing rates.

Chimeras of living flowers and silicone parts Chimeric flowers retained more of their natural attractiveness than the scented artificial flowers (compare Figs 3 and 4 relative to the real flower in each case). This provided another, more nuanced way to tease apart the roles of visual and scent signals. The large showy calyx should be responsible for much of the visual display, whereas our chemical analyses showed that the labellum and column of *D. lafleurii* are responsible for almost all of the scent bouquet, and that the mushroom-scented volatile 1-octen-3-ol is restricted to the labellum (Tables S2, S3). By utilizing real flower parts, we also included the contextual and tactile aspects of the pubescent calyces.

Flowers were dissected with a razor blade and were then combined with the complementary artificial parts (held in place by friction) to build the chimeras (Fig. 4). To control for volatiles released in response to tissue damage, the calyx of the intact flower was inconspicuously cut near the adnation of the sepal end. We used a fully crossed design, in which the four treatments included every combination of living and artificial flower parts: a real

Table 1 Summary of field experiments with *Dracula lafleurii* including hypotheses tested and response variables

Experiment	Deconstruction (Fig. 2)	Reconstruction (Fig. 3)	Chimeras (Figs 4, 5)	Visual details (Fig. 6)
Questions	Are visual or olfactory stimuli alone sufficient to attract pollinators?	Does the addition of scent to visual stimuli attract more pollinators?	Are all flower parts necessary for pollinator attraction?	How do visual patterns of the calyx affect pollinator behavior?
Experimental manipulations	Real flowers cloaked in plastic or cloth bags	Silicone flowers plus floral scent extracts	Chimeric combinations of real and silicone flower parts	Silicone flowers with differently patterned and colored calyces
Response variables	Approaches h^{-1} Lands h^{-1}	Approaches h^{-1} Lands h^{-1}	Lands h^{-1} Behavior post-landing	Lands h^{-1} Duration of visit
Statistical contrasts	Bagged treatments vs bag (negative) control Bagged treatments vs flower (positive) control	Colored vs green silicone flower Colored + scented vs colored only Colored + scented vs flower (positive) control	Real labellum chimera vs flower (positive) control Real calyx chimera vs flower (positive) control Silicone flower (no scent) vs flower (positive) control	Patterned (striped or spotted) calyx vs flower (positive) control Uniform (red or white) calyx vs flower (positive) control

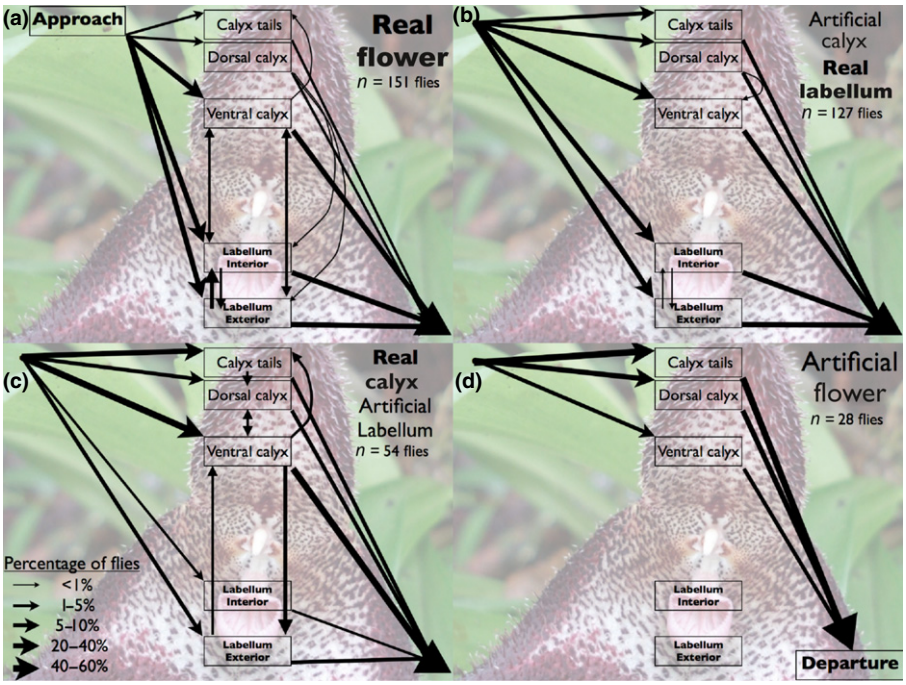

Fig. 5 Chimera treatment affects both attractions and intra-treatment transitions to *Dracula lafleurii*. The percentage of flies to make each transition within each treatment is illustrated for (a) the real flower, (b) the real labellum chimera, (c) the real calyx chimera and (d) the artificial flower. Background photograph © T. Policha.

flower, an artificial flower, a real calyx chimera (with an artificial labellum) and a real labellum chimera (with an artificial calyx). Artificial and chimeric treatments were randomly arranged around the real *D. lafleurii* plants. Our *a priori* contrasts were that each treatment had fewer visits than real flower controls (Table 1).

We also tracked how visitors moved among flower parts once they landed. These movements were tracked in kinematic diagrams to illustrate the relative frequencies of landings and transitions (Fig. 5). Preference in landing location was determined using G-tests to compare our observed distributions with the null hypothesis of no preference (equal lands to each location) (Sokal & Rohlf, 1995). In addition, we combined the first-land location data across treatments to determine what floral aspect was driving this difference in preference using combined G-tests (Sokal & Rohlf, 1995). These tests were performed in BIOMSTAT v.3.3 (Rohlf, 1999). Thirteen observation periods (6.5 h in total) were conducted between 8 and 15 February 2011.

Visual signals: the roles of contrast, pattern and color We quantified variation in coloration of *D. lafleurii* flowers using field measurements and the imaging software IMAGEJ v.1.44 (Rasband, 1997–2012) (Figs S2, S4). The unifying motif is a white background with red–maroon spots variously arranged in a linear or dispersed fashion (Figs 1, S2, S4).

To test the relative contributions of color and pattern in attracting visitors, we observed visitation rates to five treatments including four different artificial silicone flowers: a real flower, two 50% maroon/50% white artificial flowers ('spotted' and

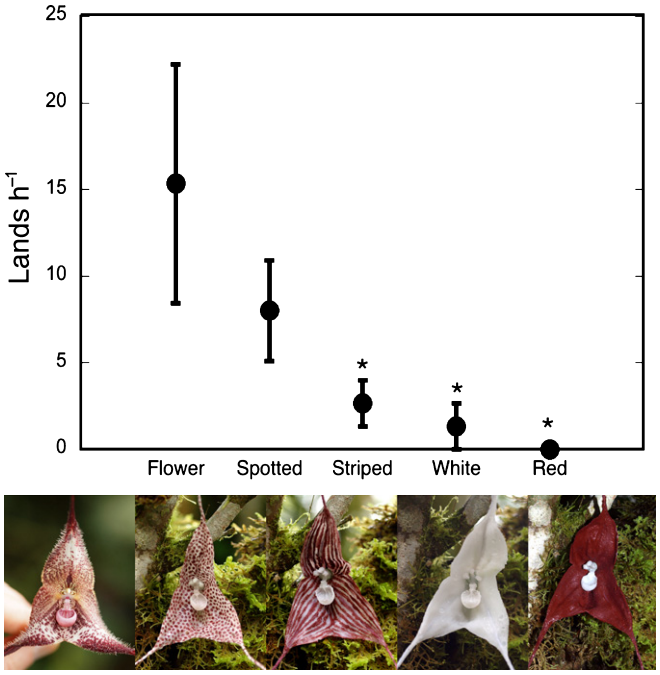

Fig. 6 To assay visual signals in *Dracula lafleurii* treatments included a real flower in the field and four artificial flowers: 50% maroon spotted, 50% maroon striped, solid white and solid red. Photos © B. A. Roy. Asterisks indicate significant *a priori* contrasts that the treatment has fewer visits than the real flower controls (*, $P \leq 0.05$). Error bars represent ± 1 SE.

'striped'), and solid 'red' and solid 'white' artificial flowers (Fig. 6; Table 1). To explicitly test the role of the visual signals, no volatiles were added to the artificial flowers in this experiment. Although the calyx color and/or pattern varied across the artificial flower treatments, the labella were fabricated in white silicone and held constant across the treatments. The *a priori* contrasts were that each treatment had fewer visits than real flower controls. Six observation periods (3 h in total) were made between 22 January and 15 February 2011.

Volatile chemistry

We analyzed the volatiles of *D. lafleurii* flowers in comparison with co-occurring mushrooms (Table S4; Fig. S5) and with other co-occurring pleurothallid orchids lacking mushroom-like labella (Fig. S6, images from Policha, 2012). Specifically, we asked: do the *Dracula* volatiles match those of fruiting fungi present in the same habitat? Do they depart substantively from the volatile bouquets of sympatric, related orchids (simultaneously controlling for habitat and phylogeny)? Whole flowers, flower parts (calyx, (lateral) petals, labellum, column) and mushrooms were extracted in 500 µl of 9 : 1 hexane : acetone for 6 h. Solvent controls were collected for each time that extracts were made, controlling for ambient scents. Analysis was performed by GC-MS.

Before analysis, all samples were filtered through quartz wool, concentrated to 50 µl under N_2 gas and 5 µl of a 0.03% toluene solution (in hexane) was added as an internal standard. Aliquots (1 µl) of the concentrated extracts were injected (splitless) into a Shimadzu GC-17A (with Shimadzu AOC-20i autoinjector), equipped with a Shimadzu QP5000 quadrupole electron impact MS (Shimadzu Corp., Kyoto, Japan) as a detector, on a highly polar ethylene glycol capillary column (EC™ Wax; W. R. Grace & Co., Columbia, MD, USA) (30 m × 0.25 mm i.d.; film thickness, 0.25 µm). Sample blends were separated using one of two temperature programs: 'temperature program 1' (30 min; exploratory, to screen all volatiles): inject at 40°C and hold for 3 min, then increase by 10°C min^{-1} to 260°C and hold for 5 min; or 'temperature program 2' (20 min; after no high-boiling compounds were found, truncated to reduce the time of analysis): inject at 40°C and hold for 3 min, then increase by 10°C min^{-1} to 200°C, then increase by 30°C min^{-1} to 260°C and hold for 9 s. The carrier gas was ultrahigh purity (99.999%) helium, with a flow rate of 1.5 ml min^{-1} (20 : 1 split ratio), and the column pressure at injection was maintained at 61 kPa.

Compounds were identified using computerized mass spectral libraries (Wiley, NIST and Adams), and verified using retention times, Kovats indices and mass spectra of authentic reference standards (Raguso *et al.*, 2006). Focusing on the 15 most common compounds, we assessed similarities among groups using nonmetric multidimensional scaling (NMDS). GC-MS total ion chromatogram peak areas were normalized by dividing by the toluene (internal standard) peak area for each sample. Normalized peak areas for the 15 most frequently encountered compounds were square root transformed and used to calculate a Bray–Curtis similarity index, employing PRIMER v.6.1.11 (Clarke & Gorley, 2006). The low stress value (0.1) indicated a close fit between the ordination and the matrix. The significance of the differences in volatile composition between *D. lafleurii* flower parts, co-occurring pleurothallid orchids and fungal species with mushroom fruiting bodies was measured using analysis of similarity (ANOSIM). The global R value for this analysis ($R = 0.579$, $P = 0.001$) indicated that sample categories were more similar internally than between defined groups, justifying further analysis of the Bray–Curtis similarity matrix. We used SIMPER as a *post-hoc* test to explore the contributions of specific compounds to differences in between-group comparisons visualized as discrete or overlapping clusters in two-dimensional Cartesian scent space resulting from NMDS, as described by Arguello *et al.* (2013). The compounds that contributed most heavily to differences between groups clustering in specific locations allowed us to insert these compounds as chemical landmarks within NMDS space, as is frequently performed in similar studies (e.g. Jürgens *et al.*, 2013).

Results

Deconstruction: both olfactory and visual cues attract floral visitors

Four-hundred and twenty flies were recorded approaching the array of bagged and unbagged flowers, 130 of which landed. Approaches to the array were dependent on treatment (Fig. 2). Individual contrasts showed differences between the 'bagged' treatments and their negative controls; the 'visual only' elicited more approaches than the 'visual control'; $t_{1,9.26} = 2.59$;

$P = 0.014$. The 'scent only' also received more approaches than the 'scent control'; $t_{1,16.64} = -1.96$; $P = 0.033$. Neither 'visual only' nor 'scent only' were approached as often as the real flower ($t_{1,9.13} = -1.86$; $P = 0.048$ and $t_{1,9.01} = 1.98$; $P = 0.040$, respectively). Neither landing rates (Fig. 2; Table S1) nor visit duration (data not shown) showed significant differences between the 'bagged' treatments and their controls, whereas landings to the real flower were significantly higher than to the 'visual only' and 'scent only' treatments ($t_{1,9.02} = -2.07$; $P = 0.034$ and $t_{1,9.01} = 2.07$; $P = 0.034$, respectively). Data available from Dryad, doi: 10.5061/dryad.7jb92 (Policha *et al.*, 2016).

Floral reconstruction: scent extracts added to a visual model stimulate landings

Approaches (Fig. 3) were significantly dependent on treatment (whole model Welch $\text{test}_{4,\ 38.97} = 6.20$; $P = 0.001$). Treatment vs control contrasts indicated that the artificial flower treatments were more attractive than their negative controls; 'visual only' vs 'material control': $t_{1,25.7} = 2.72$; $P = 0.0105$; 'visual + scent' vs 'solvent control': $t_{1,25.3} = 2.79$; $P = 0.005$. The 'visual + scent' vs 'real flower' was not significantly different ($t_{1,20.7} = 1.43$; $P = 0.084$). Landing rates (Fig. 3) were also dependent on treatment (whole model Welch $\text{test}_{4,\ 39.9} = 2.70$; $P = 0.044$); the 'visual + scent' treatment had more landings than its negative control ($t_{1,19.2} = 2.49$; $P = 0.011$). During 18 replicates (9 h in total), we observed 99 approaches resulting in 61 lands. Data available from Dryad, doi: 10.5061/dryad.7jb92 (Policha *et al.*, 2016).

Chimeras: both calyx and labellum play a role in attraction

We observed 372 visitors over 13 time replicates (6.5 h in total). Landing rates were dependent on treatment (Fig. 4, Welch $\text{test}_{3,\ 24.3} = 3.35$; $P = 0.036$). Only the 'real labellum chimera' was statistically indistinguishable from the 'real flower' ($t_{1,23.4} = -0.357$; $P = 0.362$). The unscented 'artificial flower' was visited significantly less often than the 'real flower' ($t_{1,13.4} = -2.10$; $P = 0.0275$), as was the 'real calyx chimera' ($t_{1,13.4} = -2.10$; $P = 0.0275$).

The kinematic diagrams (Fig. 5) show that visits to the 'real flower' are more complex, with more intrafloral movements than the other treatments (in particular to and from the labellum), with a substantial proportion of flies (13.3% of visitors) making these intrafloral transitions. Visitors to the unscented 'artificial flower' showed no intrafloral movement, and never landed on or moved to the labellum. The 'real labellum chimera' had more visitors than the 'real calyx chimera,' but few intrafloral transitions (1.6% of visitors), and no transitions between the calyx and the labellum. A higher proportion (14.8%) of visitors to the 'real calyx chimera' transitioned between floral organs. Further illustrating the role of the real calyx in promoting visit complexity, visit durations at the 'real flower' and the 'real calyx chimera', which were significantly longer than at the 'artificial flower' (whole model $F_{3,32} = 4.23$; $P = 0.0126$). Data available from Dryad, doi: 10.5061/dryad.7jb92 (Policha *et al.*, 2016).

Landings to the labellum dropped off precipitously in the absence of a real labellum and landings overall were dependent on its presence. The whole model combined G-test was significant ($G = 160.31$, df = 12, $P < 0.0001$), with no significant differences among the 'real flower' and 'real labellum chimera' across all floral parts ($G = 9.61$), and the 'artificial flower' and 'real calyx chimera' also across all floral parts ($G = 19.68$) (Table 2; Fig. S7).

There were significant differences across treatments in terms of preference for first-landing location (Figs 5, S7; Table 2). The treatments with real labella ('real flower' and 'real labellum chimera') elicited more landings to the labellum ('real flower': $G = 106.05$, df = 4, $P < 0.0001$; 'real labellum chimera': $G = 45.49$, df = 4, $P < 0.0001$). The treatments without real labella (the 'artificial flower' and the 'real calyx chimera') elicited more landings to the calyx ('artificial flower': $G = 37.41$, df = 4, $P < 0.0001$; 'real calyx chimera': $G = 38.03$, df = 4, $P < 0.0001$). The majority of fly visits to the 'real calyx chimera' were to the ventral surface of the calyx (closer to the labellum and the column), whereas most visits to the 'artificial flower' were to parts of the calyx (tails) most distal to the reproductive structures, with none to the labellum (Figs 5, S7; Table 2).

Visual signals: pattern is more important than color

Landing rates were dependent on treatment (whole model Kruskal–Wallis $P = 0.027$; Fig. 6). The two patterned treatments, 'spotted' and 'striped,' were visually the most similar to the 'real flower,' but only the 'spotted' treatment received the same statistical visitation rate as the 'real flower' ('spotted' vs 'real': $t_{1,25} = -1.50$; $P = 0.1805$; 'striped' vs 'real': $t_{1,25} = 2.60$; $P = 0.008$). Both the solid colored treatments received significantly fewer visits than the 'real flower' control ('white' vs 'real': $t_{1,25} = 2.87$; $P = 0.049$; 'red' vs 'real': $t_{1,25} = 3.14$; $P = 0.038$). Visit duration was also dependent on treatment (whole model Kruskal–Wallis $P = 0.037$). Visit duration was statistically significantly different for all treatments vs the 'real flower' controls ('spotted' vs 'real': $t_{1,5.1} = 2.22$; $P = 0.038$; 'striped' vs 'real':

Table 2 First-landing locations across treatments were dependent on the presence of a real labellum in *Dracula lafleurii*

Treatment	Calyx tails	Dorsal calyx	Ventral calyx	Exterior labellum	Interior labellum	Total lands per treatment
Real flower	1.99%	2.65%	25.83%	34.44%	35.10%	151
Real labellum chimera	6.30%	7.87%	23.62%	37.01%	25.20%	127
Real calyx chimera	27.78%	14.81%	48.15%	7.41%	1.85%	54
Artificial flower	53.57%	35.71%	10.71%	0%	0%	28

$t_{1,5.1} = 2.38$; $P = 0.031$; 'white' vs 'real': $t_{1,5} = 2.48$; $P = 0.028$; 'red' vs 'real': $t_{1,5} = 2.50$; $P = 0.0275$). During six replicates (3 h in total), we observed 44 visitors. Data available from Dryad, doi: 10.5061/dryad.7jb92 (Policha *et al.*, 2016).

Volatile chemistry: mushroom volatiles are novel to *Dracula* labella

Volatile bouquets differed significantly among the groups: *D. lafleurii* flower parts, mushrooms, and pleurothallid orchids (ANOSIM $R = 0.579$, $P = 0.001$) (Fig. 7; Tables S2, S3). The *Dracula* orchids have an overall scent profile that is intermediate to the other groups, sharing generic floral compounds with other pleurothallid orchids and the fungal scented 1-octen-3-ol with the mushrooms. The 1-octen-3-ol shared between mushrooms (80% of samples, $n = 20$) and *D. lafleurii* was largely restricted to the labella (71% of samples), only being detected once in a column extract (to which the labellum is adnate; 6.3% of samples). The remaining volatile compounds detected from *Dracula* extracts had different distribution patterns. Some, like benzaldehyde (labella and calyces) and 2-phenylethanol (labella and petals), were shared with the mushrooms and the other orchids (4.8% of *Dracula* samples, 55% of mushroom samples and 5.3% of pleurothallid samples; and 40% of *Dracula* samples, 40% of mushroom samples and 47% of pleurothallid samples, respectively). Others, like the generic floral volatiles benzyl alcohol (whole flowers) and methyl salicylate (columns and calyces), were common to *Dracula* and co-occurring pleurothallid orchids (71% and 47%; and 92% and 32% of samples, respectively). It appears that the presence of benzyl alcohol is one of the main features distinguishing *D. lafleurii* flowers from the mushrooms (Table S3). Finally, some compounds (3-octanone and 3-octanol) were found exclusively in the mushrooms (50% and 10% of samples, respectively), and others (linalool and its oxides, 37–42% of samples; eugenol, 32% of samples; and isoeugenol, 5.3% of samples) were restricted to the non-*Dracula* pleurothallid orchids.

When we analyzed extracts from dissected *Dracula* flowers, we found discrete volatile emission patterns between the floral organs. The labellum of *D. lafleurii* produced 1-octen-3-ol, whereas the aromatic compounds common to other orchids were more prevalent in the column, suggesting an olfactory division of labor (Tables S2, S3). The calyx was nearly scentless (small peak areas) despite being the largest (73.4% of dry mass; SE = 2.8, $n = 8$ flowers) and most conspicuously patterned floral organ. When volatiles were detected in calyces, they tended to be either volatiles indicative of plant wounding, such as (*E*)-2-hexen-1-ol and (*Z*)-3-hexen-1-ol, or shared the benzyl alcohol and methyl salicylate found in the flower's column – to which they are adnate. The lateral petals did not contribute much to the overall volatile profile (only four of 22 samples registered any volatiles), but when they did, they contained 2-phenylethanol and benzyl alcohol. Data available from Dryad, doi: 10.5061/dryad.7jb92 (Policha *et al.*, 2016).

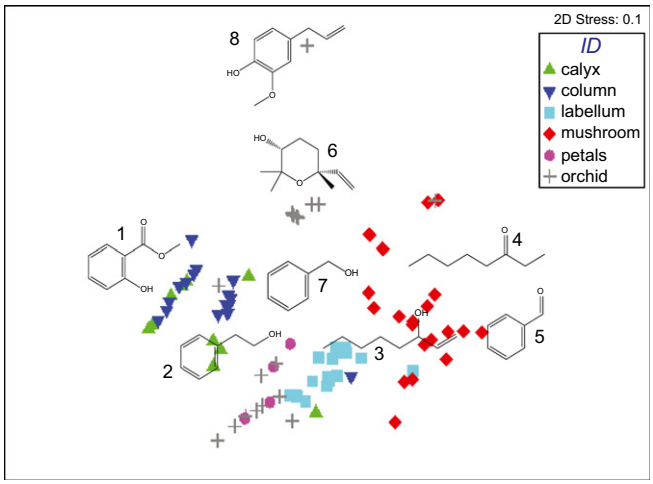

Fig. 7 Nonmetric multidimensional scaling results for the major volatile components of *Dracula lafleurii* flower parts (petals, calyx, column, labellum), co-occurring pleurothallid orchids and fungal fruiting bodies (see key). Floral tissues of *D. lafleurii* differ markedly in volatile chemistry, as the calyx and column are typified by methyl salicylate (1), the petals by 2-phenylethanol (2) and the mushroom-like labellum by 1-octen-3-ol (3), a compound common to local mushrooms at our field site, which were further characterized by 3-octanone (4), 3-octanol (not shown) and benzaldehyde (5). Linalool, its furanoid and pyranoid oxides (6), benzyl alcohol (7), eugenol (8) and isoeugenol (not shown) were emitted in varying amounts in co-occurring non-*Dracula* pleurothallid orchids. See Supporting Information Tables S2 and S3 for more details.

Discussion

Multimodal signals are synergistic and selectively favored

Our experiments demonstrate that *D. lafleurii* expresses at least two signaling modalities that are attractive to guilds of pollinating drosophilid flies (Figs 2–4, 6). By augmenting realistic artificial flowers with volatile extracts, we were able to experimentally demonstrate a synergistic effect in the signaling phenotypes (Fig. 3). Both visual and olfactory signals were sufficient to elicit approaches by flies (Figs 2, 3); however, only the 'visual + scent' treatment elicited more landings than the negative control (Fig. 3). The floral chimera experiments revealed that flies were twice as likely to land on treatments with real labella than on those with real calyces (Fig. 4), suggesting that fly attraction is primarily driven by multimodal fungal mimicry by the *Dracula* labellum.

However, the results of the color/pattern experiment indicated that visual signals also play a role in pollinator visitation to *D. lafleurii* (Fig. 6), as well as guiding fly behavior towards the labellum and column once they have landed (Fig. 5). The patterned calyx treatments ('spotted' or 'striped') elicited higher visitation rates than the solid treatments. Conspicuous spots may provide contrast at a scale that the flies can perceive, and these patterns may guide fly movement towards the floral reproductive organs, as shown previously in other examples of fly pollination (Johnson & Dafni, 1998, for example). The fact that striped and spotted patterns received statistically indistinguishable visitation rates suggests that the flies in this study do not differentiate

between them. This is interesting because both striped and spotted calyces are encountered in *Dracula* species (Fig. S8).

Two features of the volatile phenotype of *D. lafleurii* stand out. First, the compound responsible for the mushroom scent is chemically identical to a major component of the co-occurring mushrooms (Tables S2, S3). The eight-carbon volatile compound 1-octen-3-ol produced by *D. lafleurii* is the character-bearing olfactory note associated with many fungi, including cultivated *Agaricus* mushrooms (Buchbauer *et al.*, 1993; Combet *et al.*, 2006). Second, the emission of this fungal volatile is largely restricted to the only visibly mushroom-like part of the *Dracula* flower, the gilled labellum (Figs 1, S1; Tables S2, S3). In addition, by sampling other co-occurring and closely related orchids (*Masdevallia* spp. etc.; Fig. S6; Table S2), we demonstrated that this volatile was unique to the *Dracula* species at our field site (see also Policha, 2014). When produced by fungi, 1-octen-3-ol and other eight-carbon compounds are known to attract both fungivorous and fungivore-predacious species (Pierce *et al.*, 1991; Faldt *et al.*, 1999; Combet *et al.*, 2006), although both concentration and enantiomeric configuration seem to influence the effects on behavior (Cammaerts & Mori, 1987). Although we did not augment artificial flowers or parts with specific volatile compounds, we did account for the actual chemical phenotype of *D. lafleurii* by using labellum extracts rather than applying synthetic compounds. Furthermore, we have determined the enantiomeric configuration of 1-octen-3-ol from the labella of *D. lafleurii* to be the R-(–) enantiomer, which also was present in our co-occurring mushroom samples (see Methods S1) and is the dominant enantiomer of many fungal species (Zawirska-Wojtasiak, 2004). 1-Octen-3-ol has also been implicated in herbivore defense, and may play other ecological roles in *D. lafleurii*, in addition to pollinator attraction (Wood *et al.*, 2001).

The ability to attract a diverse assemblage of mushroom-associated flies may be beneficial, given the often unpredictable, patchy distributions and ephemeral nature of fleshy mushrooms. We know that flowers of *D. lafleurii* are visited by at least 17 different cryptic species of small drosophilid flies (Policha, 2014). These diverse visitors, at least 11 species of which have been caught with pollinia (Endara *et al.*, 2010; Policha, 2014), may have distinct life history strategies and distinct perceptual biases, and may be responding to different aspects of the floral phenotype, such as the showy calyx (Leonard *et al.*, 2011). Unfortunately, we cannot differentiate these small flies in the field. However, our experiments reveal that the mushroom-scented labellum entices flies to approach and land, whereas the calyx markings cause them to behave differently (e.g. to stay longer, or to move between flower parts) once they are there.

Chimeric flowers decouple larger advertising from close range handling

The use of chimeric flowers allowed us to experimentally determine the roles of each major floral component (calyx vs labellum) in pollinator attraction. Although overall visitation was driven by the presence of a real, scented labellum (Figs 4, 5, S7), visits to treatments with real calyces lasted longer and included more complex behavior, specifically with more intrafloral movements (Fig. 5). We know that the olfactory component of floral display is restricted largely to the labellum (mushroom volatiles) or to the adjacent column (floral aromatic volatiles) (Tables S2, S3), with the calyces providing much of the visual stimuli. The 'real calyx chimera' presented primarily the visual signals of *D. lafleurii*, whereas the real labellum chimera presented both visual and olfactory signals. The fact that the pairing of the visual aspect of the artificial calyx with a real, scented labellum ('real labellum chimera') produced significantly higher visitation rates than the real calyx without the scented labellum ('real calyx chimera') further supports a synergistic effect.

The role of the labellum in increasing visitation to any part of the flower suggests that scent is an important attractant over a longer distance, but that attributes of the calyx may be important for close range orientation, intrafloral movements or visitor retention. *Drosophila melanogaster* has been shown to require visual feedback for odor-guided food localization (Frye *et al.*, 2003), and this may also be occurring in these related flies. As our artificial calyces included visual patterns, this suggests a tactile or gustatory/contact chemoreceptive component of visitor retention that we were unable to address in the current study. The real calyces are also variously pubescent, possibly focusing visitors towards the column or otherwise promoting longer visit durations.

Although these flowers are not as visitor limited as many orchids (Tremblay *et al.*, 2005), they still experience fertilization rates that are quite low (5.7% of *D. lafleurii* flowers set fruit under natural conditions; $n = 35$; Policha, 2014). Traits that promote longer visits or more intrafloral movements should increase the likelihood of pollinia removal in this species and should be selectively favored.

Exploitation of perceptual biases

Dracula flowers have long been suspected of being Batesian mimics (Vogel, 1978), in part because of the lack of a visible nectar reward. The absence of reducing sugars in *Dracula* flowers was confirmed by Endara *et al.* (2010) in *D. lafleurii* using Combur® urinalysis strips. However, mushroom-visiting flies may not be expected to respond to sugar as a reward. Instead, other resources (or the mimicry thereof) that are more consistent with their life histories might serve as a better reward, such as food microorganisms (yeast), shelter, brood or lekking sites. Floral variability is predicted in Batesian or deceptive mimicry systems as a way to impair learning by duped visitors (Moya & Ackerman, 1993; Roy & Widmer, 1999; Salzmann *et al.*, 2007), and *D. lafleurii* shows considerable variation in both size (calyx area, 411–1623 mm^2; labellum area, 16–97 mm^2) and the amount of pigment in color patterns (percentage pigmentation in the calyx, 38–93%; $n = 22$ flowers; Figs S2, S4). This variation is also predicted because of the generalized nature of the mimicry; in the absence of a specific model, there are a range of phenotypes that may be considered attractive.

Another plausible role for the spotted calyx (Figs 1, 6, S2) is to exploit the lekking behavior (Grimaldi, 1986; Burla, 1990) of

these flies by presenting fly decoys. Both intra- and interspecific aggregations are common in related dipteran taxa (Jaenike & James, 1991; Jaenike *et al.*, 1992), and the dark spots on a light background may look like other flies, serving to attract additional individuals (Johnson & Midgley, 1997). We occasionally observed 'swarming' (> 180 visits h^{-1}), in which large groups of flies aggregate, mate and engage in territorial competition at *Dracula* flowers. We have observed this same phenomenon in *Zygothrica* spp. flies visiting actual mushrooms at our field site. Further work is needed to determine how striped and spotted patterns trigger bias in fly's visual, gustatory or tactile senses. This is important beyond the *Dracula* system, because many fly-pollinated flowers across a range of brood-site mimicry strategies (and dipteran taxa) are spotted or striped (Atwood, 1985; Miyake & Yafuso, 2003; Burgess *et al.*, 2004; Ollerton *et al.*, 2009).

The volatile bouquet of *D. lafleurii*, whilst possessing components of co-occurring orchid and mushroom profiles, did not completely overlap with either group (Fig. 7). Unique to the pleurothallid orchids were the floral volatiles eugenol and linalool, and unique to the mushroom profile were the fungal volatiles 3-octanol and 3-octanone. In mushrooms, 1-octen-3-ol is the most abundant eight-carbon fungal volatile (Combet *et al.*, 2006), and may function in part as a signaling molecule, correlated with events such as sporulation (Faldt *et al.*, 1999) and spore germination (Chitarra *et al.*, 2004). In terms of prevalence and abundance, 1-octen-3-ol is a key element of the fungal volatile motif (Tressl *et al.*, 1982; Combet *et al.*, 2006), and is therefore predicted to be a focus of signal convergence in the evolution of fungal mimicry in co-occurring flowers.

Our floral chimera experiment (Figs 4, 5) revealed that the integration of visual and olfactory signals from different flower parts is critical to the success of *D. lafleurii* in attracting drosophilid flies. Similar phenomena have been documented in other pollination systems, in which each component elicits a subset of necessary behaviors, but both are required for complete pollinator function (Raguso & Willis, 2002; Leonard *et al.*, 2011). Recent studies have explored the degree to which pollinator-mediated selection drives the functional integration of different components of floral display (Ordano *et al.*, 2008; Rosas-Guerrero *et al.*, 2011). For example, Armbruster *et al.* (2005) experimentally dissected a subtle morphometric relationship governing pollen deposition by megachilid bees visiting *Dalechampia ipomoeafolia* blossoms. Path analysis revealed that stigma pollen loads were significantly impacted by interactions between floral bract length (visual attractant), resin gland area (floral reward) and resin gland–stigma distance, functionally integrating ethological and mechanical aspects with pollinator behavior. Our experimental results suggest that a similar relationship should govern the removal and transfer of pollinaria in flowers of *D. lafleurii* and similar species. The integration of multimodal stimuli is commonly observed by insects when experiments include more than one sensory modality (Leonard & Masek, 2014). Studies of *Drosophila melanogaster* have demonstrated that combined visual–olfactory conditioning increases their learning ability, even near the threshold of stimulus detection (Guo & Guo, 2005). The drosophilid flies that pollinate *D. lafleurii* in Ecuador are new to science and several await formal taxonomic description. However, it is clear from our studies that they, too, utilize multimodal foraging behavior to find and visit *Dracula* flowers.

What is so remarkable in *Dracula* flowers is the cross-kingdom mimicry in both visual and chemical signals, leading to a novel and successful niche for these orchids. Without using the highly detailed 3D-printing to produce the silicone flowers it would not have been possible to so clearly show that the labellum is responsible for the mushroom motif, with both a visual and olfactory fungal phenotype, or to demonstrate a role for the large, showy calyx. Our findings suggest that the novel synapomorphic characters that define the genus *Dracula* – a mushroom-like labellum, and a showy, patterned calyx – work together synergistically to enhance pollinator attraction by exploiting the visual and chemosensory perceptual biases of drosophilid flies. 3D printing provides an effective tool for experimental manipulation of complex morphological traits not conducive to gene silencing or selective breeding in nonmodel systems, such as epiphytic tropical orchids.

Acknowledgements

Thanks are due to the staff at Reserva los Cedros (www.reservaloscedros.org), especially J. Decoux, as well as the volunteers who helped to collect data, particularly G. Baily-Testa, E. Cantor, J. Digby, E. Heikkila, R. Igo, E. Scriven, M. Wherley and F. Von Bechtolsheim. For assistance in the field, we also thank R. Manobanda and J. McAlpine. We thank Marsh Hollow Orchids, J&L Orchids, as well as the Jardín Botánico Lankester and The Monteverde Orchid Garden in Costa Rica for access to their collections during method development, A. Gaskett for help with GC-MS protocols, the Cresko laboratory for ImageJ consultation and D. Grimaldi for help in identifying our pollinators. We are grateful to our collaborators at the Museo Ecuatoriano de Ciencias Naturales (M. Altamirano, R. Batallas, D. Fernández Fernández) and the Escuela Politécnica Nacional (L. Albuja, V. Carvajal López, L. Espinoza, A. Troya) for helping to obtain the necessary permits to work in Ecuador (no. 001-07 IC-F-DRCI-MA, no. 02-10-IC-FLO-DPAI/MA, no. 03-09-IC-FAU-DPAI/MA, no. 07-2010-IC-FAU-DPAI/MA, no. 03-2011-IC-FLO-DPAI/MA). Funding was provided by the National Geographic Society (no. 8317-07 to B.A.R. and B.T.M.D.) and the National Science Foundation (NSF) (DEB-0841613 to B.A.R. and B.T.M.D.). GC-MS analysis at Cornell was supported by NSF grant DEB-0746106 to R.A.R. We also thank three anonymous reviewers for providing comments that improved the manuscript.

Author contributions

T.P., A.D., M.B., B.T.M.D., R.A.R. and B.A.R. planned and designed the research. M.B. constructed the artificial flowers. T.P., A.D., M.B. and B.A.R. performed the experiments. T.P., A.D., M.B., B.T.M.D. and B.A.R. conducted the fieldwork. T.P., R.A.R. and B.A.R. analyzed the data. T.P., M.B., B.T.M.D., R.A.R. and B.A.R. wrote the manuscript.

References

Arguello JR, Sellanes C, Lou YR, Raguso RA. 2013. Can yeast (*S. cerevisiae*) metabolic volatiles provide polymorphic signaling? *PLoS ONE* **8**: e70219.

Armbruster WS, Antonsen L, Pélabon C. 2005. Phenotypic selection on *Dalechampia* blossoms: honest signaling affects pollination success. *Ecology* **86**: 3323–3333.

Atwood JT. 1985. Pollination of *Paphiopedilum rothschildianum*: brood-site deception. *National Geographic Research Spring* **1985**: 247–254.

Balogh ACV, Gamberale-Stille G, Leimar O. 2008. Learning and the mimicry spectrum: from quasi-Bates to super-Müller. *Animal Behaviour* **76**: 1591–1599.

Barth FG. 1985. *Insects and flowers: the biology of a partnership*. Princeton, NJ, USA: Princeton University Press.

Bierzychudek P. 1981. *Asclepias, Lantana*, and *Epidendrum* – a floral mimicry complex. *Biotropica* **13**: 54–58.

Blanco M, Barboza G. 2005. Pseudocopulatory pollination in *Lepanthes* (Orchidaceae: Pleurothallidinae) by fungus gnats. *Annals of Botany* **95**: 763–772.

Buchbauer G, Jirovetz L, Wasicky M, Nikiforov A. 1993. The aroma of edible mushrooms – headspace analysis using GC FID and GC FTIR MS. *Zeitschrift fur Lebensmittel-Untersuchung und -Forschung* **197**: 429–433.

Burgess KS, Singfield J, Melendez V, Kevan PG. 2004. Pollination biology of *Aristolochia grandiflora* (Aristolochiaceae) in Veracruz, Mexico. *Annals of the Missouri Botanical Garden* **91**: 346–356.

Burla H. 1990. Lek behavior in hypercephalic *Zygothrica dispar* Wiedemann (Diptera, Drosophilidae). *Journal of Zoological Systematics & Evolutionary Research* **28**: 69–77.

Cammaerts MC, Mori K. 1987. Behavioural activity of pure chiral 3-octanol for the ants *Myrmica scabrinodis* Nyl. and *Myrmica rubra* L. *Physiological Entomology* **12**: 381–385.

Campos EO, Bradshaw HD, Daniel TL. 2015. Shape matters: corolla curvature improves nectar discovery in the hawkmoth *Manduca sexta*. *Functional Ecology* **29**: 462–468.

Chitarra GS, Abee T, Rombouts FM, Posthumus MA, Dijksterhuis J. 2004. Germination of *Penicillium paneum* conidia is regulated by 1-octen-3-ol, a volatile self-inhibitor. *Applied and Environmental Microbiology* **70**: 2823–2829.

Chittka L, Spaethe J, Schmidt A, Hickelsberger A. 2001. Adaptation, constraint, and chance in the evolution of flower color and pollinator color vision. In: Chittka L, Thomson JD, eds. *Cognitive ecology of pollination*. Cambridge, UK: Cambridge University Press, 106–126.

Clarke K, Gorley R. 2006. *Primer v6: user manual/tutorial*. Plymouth, UK: Primer-E.

Combet E, Henderson J, Eastwood DC, Burton KS. 2006. Eight-carbon volatiles in mushrooms and fungi: properties, analysis, and biosynthesis. *Mycoscience* **47**: 317–326.

Cozzolino S, Widmer A. 2005. Orchid diversity: an evolutionary consequence of deception? *Trends in Ecology & Evolution* **20**: 487–494.

Dafni A. 1984. Mimicry and deception in pollination. *Annual Review of Ecology and Systematics* **15**: 259–278.

Dentinger BTM, Roy BA. 2010. A mushroom by any other name would smell as sweet: *Dracula* orchids. *McIlvainea* **19**: 1–13.

Dethier VG. 1976. *The hungry fly: a physiological study of the behavior associated with feeding*. Cambridge, MA, USA: Harvard University Press.

Ellis AG, Johnson SD. 2010. Floral mimicry enhances pollen export: the evolution of pollination by sexual deceit outside of the Orchidaceae. *American Naturalist* **176**: E143–E151.

Endara L, Grimaldi DA, Roy BA. 2010. Lord of the flies: pollination of *Dracula* orchids. *Lankesteriana* **10**: 1–11.

Faldt J, Jonsell M, Nordlander G, Borg-Karlson AK. 1999. Volatiles of bracket fungi *Fomitopsis pinicola* and *Fomes fomentarius* and their functions as insect attractants. *Journal of Chemical Ecology* **25**: 567–590.

Frye MA, Tarsitano M, Dickinson MH. 2003. Odor localization requires visual feedback during free flight in *Drosophila melanogaster*. *Journal of Experimental Biology* **206**: 843–855.

Gaskett AC. 2011. Orchid pollination by sexual deception: pollinator perspectives. *Biological Reviews* **86**: 33–75.

Goodrich KR, Zjhra ML, Ley CA, Raguso RA. 2006. When flowers smell fermented: the chemistry and ontogeny of yeasty floral scent in pawpaw (*Asimina triloba:* Annonaceae). *International Journal of Plant Sciences* **167**: 33–46.

Grimaldi D. 1986. *Phylogenetics and taxonomy of* Zygothrica *(Diptera: Drosophilidae)*. PhD thesis, Cornell University, Ithaca, New York, NY, USA.

Grimaldi DA. 1987. Phylogenetics and taxonomy of *Zygothrica* Diptera Drosophilidae. *Bulletin of the American Museum of Natural History* **186**: 103–268.

Guo J, Guo A. 2005. Crossmodal interactions between olfactory and visual learning in *Drosophila*. *Science* **309**: 307–310.

IBM Corp. 2013. *SPSS v22 statistics for Macintosh*. Armonk, NY, USA: IBM Corp.

Jaenike J. 1978. Host selection by mycophagous *Drosophila*. *Ecology* **59**: 1286–1288.

Jaenike J, Bartelt RJ, Huberty AF, Thibault S, Libler JS. 1992. Aggregations in mycophagous *Drosophila* (Diptera, Drosophilidae) – candidate pheromones and field responses. *Annals of the Entomological Society of America* **85**: 696–704.

Jaenike J, James AC. 1991. Aggregation and the coexistence of mycophagous *Drosophila*. *Journal of Animal Ecology* **60**: 913–928.

Jersáková J, Johnson SD, Kindlmann P. 2006. Mechanisms and evolution of deceptive pollination in orchids. *Biological Reviews* **81**: 219–235.

Johnson SD, Dafni A. 1998. Response of bee-flies to the shape and pattern of model flowers: implications for floral evolution in a Mediterranean herb. *Functional Ecology* **12**: 289–297.

Johnson SD, Midgley JJ. 1997. Fly pollination of *Gorteria diffusa* (Asteraceae), and a possible mimetic function for dark spots on the capitulum. *American Journal of Botany* **84**: 429–436.

Jorgensen PM, León-Yanez S, eds. 1999. *Catalogue of the vascular plants of Ecuador*. St Louis, MO, USA: Missouri Botanical Garden Press.

Jürgens A, Wee S-L, Shuttleworth A, Johnson SD. 2013. Chemical mimicry of insect oviposition sites: a global analysis of convergence in angiosperms. *Ecology Letters* **16**: 1157–1167.

Kaiser R. 1993. On the scent of orchids. In: Teranishi R, Buttery RG, Sugisawa H, eds. *Bioactive volatile compounds from plants*. Washington, DC, USA: American Chemical Society, 240–268.

Kaiser R. 2006. Flowers and fungi use scents to mimic each other. *Science* **311**: 806–807.

Larson BMH, Kevan PG, Inouye DW. 2001. Flies and flowers: taxonomic diversity of anthophiles and pollinators. *Canadian Entomologist* **133**: 439–465.

Leonard AS, Dornhaus A, Papaj DR. 2011. Forget-me-not: complex floral displays, inter-signal interactions, and pollinator cognition. *Current Zoology* **57**: 215–224.

Leonard A, Masek P. 2014. Multisensory integration of colors and scents: insights from bees and flowers. *Journal of Comparative Physiology A* **200**: 463–474.

Luer CA. 1993. *Icones* Pleurothallidinarum *X. Systematics of* Dracula *(Orchidaceae)*. St Louis, MO, USA: Missouri Botanical Garden.

McAlpine J. 2013. *The role of yeasts in the pollination success of a Neotropical orchid*. Master's thesis, University of Oregon, Eugene, OR, USA.

McNeil L 2013. *Mimicry: The art of deception among orchids. National Geographic News*. [WWW document] URL http://video.nationalgeographic.com/video/news/orchid-mimicry-ngoa [accessed 4 December 2015].

Menzel R. 1975. Colour receptors in insects. In: Horridge GA, ed. *The compound eye and vision of insects*. Oxford, UK: Clarendon Press, 121–153.

Mesler MR, Ackerman JD, Lu KL. 1980. The effectiveness of fungus gnats as pollinators. *American Journal of Botany* **67**: 564–567.

Miyake T, Yafuso M. 2003. Floral scents affect reproductive success in fly-pollinated *Alocasia odora* (Araceae). *American Journal of Botany* **90**: 370–376.

Moré M, Cocucci AA, Raguso RA. 2013. The importance of oligosulfides in the attraction of fly pollinators to the brood-site deceptive species *Jaborosa rotacea* (Solanaceae). *International Journal of Plant Sciences* **174**: 863–876.

Moya S, Ackerman JD. 1993. Variation in the floral fragrance of *Epidendrum ciliare* (Orchidaceae). *Nordic Journal of Botany* **13**: 41–47.

Myers N, Mittermeier RA, Mittermeier CG, da Fonseca GAB, Kent J. 2000. Biodiversity hotspots for conservation priorities. *Nature* **403**: 853–858.

Ollerton J, Masinde S, Meve U, Picker M, Whittington A. 2009. Fly pollination in *Ceropegia* (Apocynaceae: Asclepiadoideae): biogeographic and phylogenetic perspectives. *Annals of Botany* **103**: 1501–1514.

Ordano M, Fornoni J, Boege K, Domínguez CA. 2008. The adaptive value of phenotypic floral integration. *New Phytologist* **179**: 1183–1192.

Papadopulos AST, Powell MP, Pupulin F, Warner J, Hawkins JA, Salamin N, Chittka L, Williams NH, Whitten WM, Loader D *et al.* 2013. Convergent evolution of floral signals underlies the success of Neotropical orchids. *Proceedings of the Royal Society B: Biological Sciences* **280**: 20130960.

Peck M, Tirira D, Mariscal A, Paredes K. 2008. *Developing a sustainable network for primates in Ecuador (PRIMENET): Darwin Initiative final report.* Brighton, UK: University of Sussex.

Peter CI, Johnson SD. 2008. Mimics and magnets: the importance of color and ecological facilitation in floral deception. *Ecology* **89**: 1583–1595.

Pierce AM, Pierce HD, Borden JH, Oehlschlager AC. 1991. Fungal volatiles: semiochemicals for stored-product beetles (Coleoptera, Cucujidae). *Journal of Chemical Ecology* **17**: 581–597.

Policha T. 2012. *Plantas de Mindo: una guía de bosque nublado del Chocó Andino/ Plants of Mindo: a guide to the cloud forest of the Andean Choco.* Eugene, OR, USA: American Herbal Dispensary Press.

Policha T. 2014. *Pollination biology of the mushroom-mimicking orchid genus* Dracula. PhD thesis, University of Oregon, Eugene, OR, USA.

Policha T, Davis A, Barnadas M, Dentinger BTM, Raguso RA, Roy BA. 2016. Data from: disentangling visual and olfactory signals in mushroom-mimicking *Dracula* orchids using realistic 3D printed flowers. *New Phytologist.* doi: 10.5061/dryad.7jb92.

Pridgeon AM, Solano R, Chase MW. 2001. Phylogenetic relationships in *Pleurothallidinae* (Orchidaceae): combined evidence from nuclear and plastid DNA sequences. *American Journal of Botany* **88**: 2286–2308.

Raguso RA. 2006. Behavioral responses to floral scent: experimental manipulations and the interplay of sensory modalities. In: Dudareva N, Pichersky E, eds. *The biology of floral scent.* Boca Raton, FL, USA: CRC Press, 297–319.

Raguso RA, Schlumpberger BO, Kaczorowski RL, Holtsford TP. 2006. Phylogenetic fragrance patterns in *Nicotiana* sections *Alatae* and *Suaveolentes*. *Phytochemistry* **67**: 1931–1942.

Raguso RA, Willis MA. 2002. Synergy between visual and olfactory cues in nectar feeding by naive hawkmoths, *Manduca sexta*. *Animal Behaviour* **64**: 685–695.

Rasband WS. 1997–2012. *ImageJ 1.44.* Bethesda, MD, USA: US National Institutes of Health.

Rohlf FJ. 1999. *BIOMstat 3.3.* Setauket, NY, USA: Exeter Software.

Rosas-Guerrero V, Quesada M, Armbruster WS, Pérez-Barrales R, Smith SD. 2011. Influence of pollination specialization and breeding system on floral integration and phenotypic variation in *Ipomoea*. *Evolution* **65**: 350–364.

Roy BA, Widmer A. 1999. Floral mimicry: a fascinating yet poorly understood phenomenon. *Trends in Plant Science* **4**: 325–330.

Ruxton GD, Schaefer HM. 2011. Alternative explanations for apparent mimicry. *Journal of Ecology* **99**: 899–904.

Salzmann CC, Nardella AM, Cozzolino S, Schiestl FP. 2007. Variability in floral scent in rewarding and deceptive orchids: the signature of pollinator-imposed selection? *Annals of Botany* **100**: 757–765.

Schaefer HM, Ruxton GD. 2009. Deception in plants: mimicry or perceptual exploitation? *Trends in Ecology & Evolution* **24**: 676–685.

Schiestl FP. 2005. On the success of a swindle: pollination by deception in orchids. *Naturwissenschaften* **92**: 255–264.

Schiestl FP, Dötterl S. 2012. The evolution of floral scent and olfactory preferences in pollinators: coevolution or pre-existing bias? *Evolution* **66**: 2042–2055.

Sierra R, ed. 1999. *Propuesta preliminar de un sistema de clasificación de vegetación para el Ecuador continental.* Quito, Ecuador: Proyecto INEFAN/GEF-BIRF y EcoCiencia.

Smithson A. 2002. The consequences of rewardlessness in orchids: reward-supplementation experiments with *Anacamptis morio* (Orchidaceae). *American Journal of Botany* **89**: 1579–1587.

Sokal RR, Rohlf FJ. 1995. *Biometry.* New York, NY, USA: W. H. Freeman & Co.

Sprengel CK. 1793. *Das entdeckte geheimnis der natur im bau und in der befruchtung der blumen (The secret of nature in the form and fertilisation of flowers discovered).* Berlin, Germany: F. Vieweg.

Ssymank A, Kearns CA, Pape T, Thompson C. 2008. Pollinating flies (Diptera): a major contribution to plant diversity and agricultural production. *Tropical Conservancy* **9**: 86–89.

Stensmyr MC, Urru I, Collu I, Celander M, Hansson BS, Angioy A-M. 2002. Rotting smell of dead-horse arum florets. *Nature* **420**: 625–626.

Stokl J, Strutz A, Dafni A, Svatos A, Doubsky J, Knaden M, Sachse S, Hansson BS, Stensmyr MC. 2010. A deceptive pollination system targeting drosophilids through olfactory mimicry of yeast. *Current Biology* **20**: 1846–1852.

Streitel SG. 2009. *Fluorescent pigments (daylight). Kirk-Othmer encyclopedia of chemical technology.* Chichester, UK: John Wiley & Sons. doi: 10.1002/0471238961.0612211519201805.a01.

The Plant List. 2013. *The Plant List: a working list of all plant species. Version 1.1.* [WWW document] URL http://www.theplantlist.org [accessed 4 December 2015].

Tremblay RL, Ackerman JD, Zimmerman JK, Calvo RN. 2005. Variation in sexual reproduction in orchids and its evolutionary consequences: a spasmodic journey to diversification. *Biological Journal of the Linnean Society* **84**: 1–54.

Tressl R, Bahri D, Engel KH. 1982. Formation of eight-carbon and ten-carbon components in mushrooms (*Agaricus campestris*). *Journal of Agricultural and Food Chemistry* **30**: 89–93.

Van der Cingel NA. 2001. *An atlas of orchid pollination – America, Africa, Asia and Australia.* Rotterdam, the Netherlands: A. A. Balkema.

Van der Niet T, Hansen DM, Johnson SD. 2011. Carrion mimicry in a South African orchid: flowers attract a narrow subset of the fly assemblage on animal carcasses. *Annals of Botany* **107**: 981–992.

Van der Pijl L, Dodson CH. 1966. *Orchid flowers: their pollination and evolution.* Coral Gables, FL, USA: University of Miami Press.

Vereecken NJ, McNeil JN. 2010. Cheaters and liars: chemical mimicry at its finest. *Canadian Journal of Zoology* **88**: 725–752.

Vogel S. 1978. Pilzmückenblumen als Pilzmimeten (Fungus-gnat flowers mimicking fungi). *Flora (Germany)* **167**: 329–398.

Wood WF, Archer CL, Largent DL. 2001. 1-Octen-3-ol, a banana slug antifeedant from mushrooms. *Biochemical Systematics and Ecology* **29**: 531–533.

Zawirska-Wojtasiak R. 2004. Optical purity of (R)-(–)-1-octen-3-ol in the aroma of various species of edible mushrooms. *Food Chemistry* **86**: 113–118.

Supporting Information

Additional supporting information may be found in the online version of this article.

Fig. S1 Co-occurring mushrooms: illustrating the visual similarity of *Dracula lafleurii*.

Fig. S2 The variation in size and coloration of *Dracula lafleurii*, with four examples.

Fig. S3 The methods and results of the bioassays performed with UV pigments, demonstrating that UV does not play a strong role in attraction to *Dracula lafleurii*.

Fig. S4 An example of how contrasting patterns were measured in *Dracula lafleurii* using ImageJ.

Fig. S5 Mushroom specimens included in the volatile analysis.

Fig. S6 Pleurothallid orchid species included in the volatile analysis.

Fig. S7 Four frequency graphs illustrating that preference for landing location depends on treatment in real, artificial and chimeric flowers.

Fig. S8 Photograph of *Dracula vampira* (Luer) Luer.

Table S1 Summary of field experiments including hypotheses tested, response variables and results

Table S2 Number of samples (dissected *Dracula* flowers, mushrooms, other pleurothallid orchids) containing the 15 most common volatiles, including retention times on a polar ethylene glycol capillary column and Kovats indices

Table S3 SIMPER analysis output of GC-MS data

Table S4 Mushroom specimens collected for scent analysis

Methods S1 Chiral GC-MS analysis to identify which enantiomer of 1-octen-3-ol is produced by the labellum.

Please note: Wiley Blackwell are not responsible for the content or functionality of any supporting information supplied by the authors. Any queries (other than missing material) should be directed to the *New Phytologist* Central Office.