New Phytologist

***New Phytologist* Supporting Information Figs S1–S8, Tables S1, S2 & S4 and Methods S1**

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

Authors: Tobias Policha, Aleah Davis, Melinda Barnadas, Bryn T. M. Dentinger, Robert A. Raguso and Bitty A. Roy.

Article acceptance date: 1 December 2015

The following Supporting Information is available for this article:

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

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

Fig. S3 Figure illustrating the methods and results of the bioassays done with ultra violet 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 Photo of *Dracula vampira* (Luer) Luer.

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

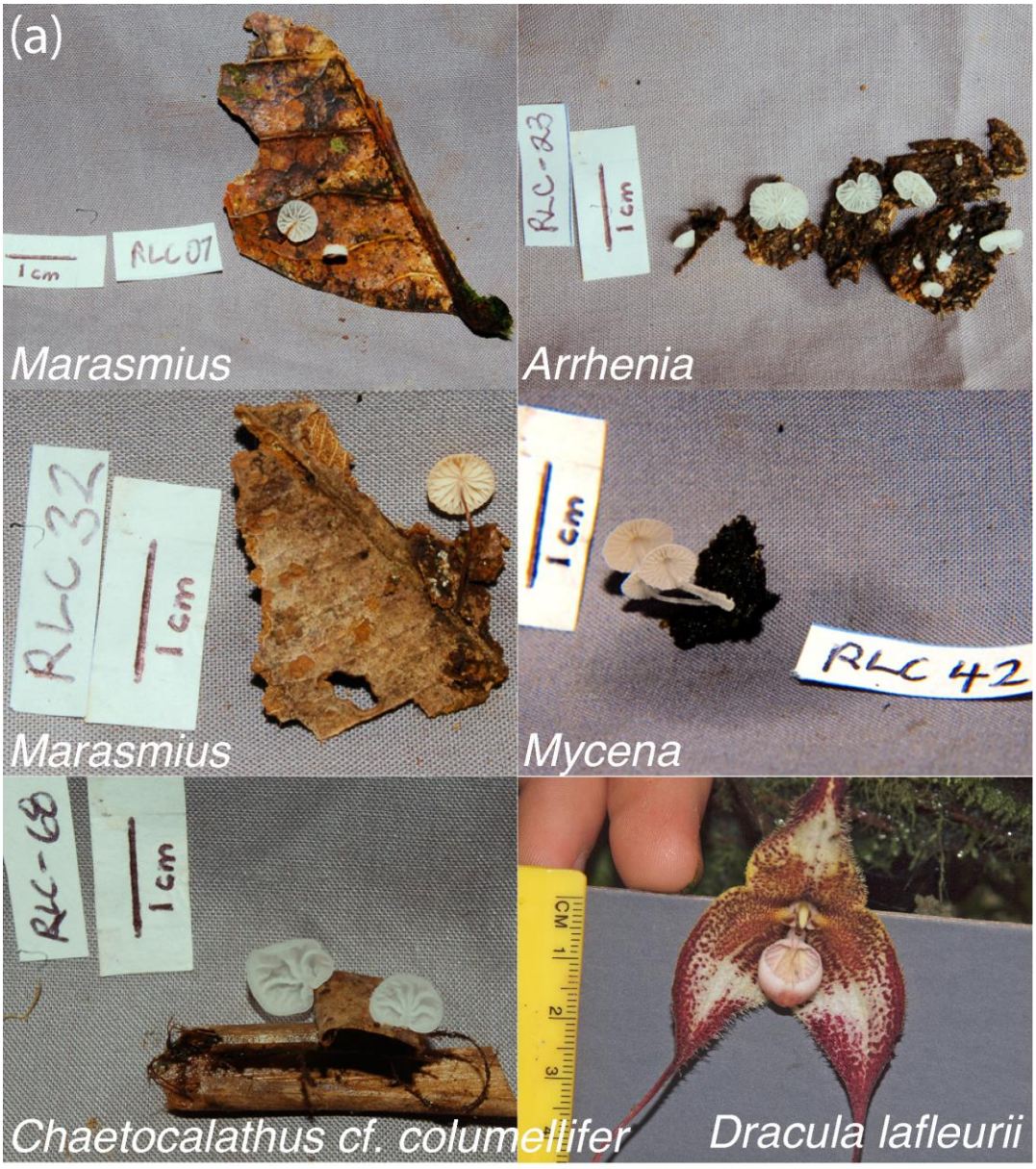
Table S2 Showing the number of samples (dissected *Dracula* flowers, mushrooms, other pleurothallid orchids) containing the 15 most common volatiles, including retention times on a polar ethylene glycol capillary column and Kovats indices

Table S3 SIMPER analysis output of GC-MS data (separate Excel file)

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.

Fig. S1 (a, b) Co-occurring mushrooms: Illustrating the visual similarity of *Dracula lafleurii*. Almost half (44%) of the mushroom species (*n* = 343) we collected in the cloud forest at Reserva Los Cedros had pilei in the ~1cm range, remarkably similar in size to *D. lafleurii* labella. (a) Collection numbers RLC 07 – RLC 68 photos © B.T.M. Dentinger. (b) Collection numbers RLC 168 – RLC 301 photos © M. Wherley, RLC 705 photo © J. McAlpine. Both photos of *D. lafleurii* © B.A. Roy. All fungal specimens deposited at Kew Royal Botanical Gardens (K) and the Herbario Nacional de Ecuador (QCNE).




(b)
Gymnopus
RLC162
1cm
RLC191
1cm
Mycena
Marasmiaceae
RLC263
1cm
RLC301
1cm
Mycena
LABORATORY
SCIENTIFIC INSTRUMENTS & CHEMI
RLC705
Coprinus
CM
Dracula lafleurii

Fig. S2 Variation in size and coloration of *Dracula lafleurii*. The experimental treatments manipulating color and pattern were based on the natural variation present in the flowers, some of which is illustrated here. Upper left: (flower BRL3.1) sepal area = 530 mm^2; 42.9% pigmented; labellum area = 30.6mm^2. Upper right (flower BRL2.2) sepal area = 1442.7 mm^2; 39.0% pigmented; labellum area =76.176mm^2. Lower left (flower RLC2.8) sepal area = 411.49 mm^2; 66.7% pigmented; labellum area = 46.71 mm^2. Lower right (flower RLC3.4) sepal area = 1303.1 mm^2; 81.9% pigmented; labellum area = 44.22 mm^2. Across 22 flowers sepal area ranged from 411 to 1623 mm^2; pigmentation ranged from 38 to 93%; and labella area ranged from16 to 97mm^2. Photos © B. A. Roy.

Fig. S3 UV does not play a strong role in attraction to *Dracula lafleurii*. (a) To measure spectral reflectance (300–700 nm) we used a USB4000 miniature fiber optic spectrometer with a T300-RT-UV-VIS probe (Ocean Optics, Dunedin, FL, USA). We present the average of five readings per part of the flower (column, exterior surface of the labellum, surface interior of the labellum, central (ventral) calyx, distal (ventral) calyx and leaves). Our measurements of UV reflectance do not indicate a strong UV signal in *D. lafleurii*. What little UV reflectance there is appears to come primarily from the column. Both surfaces of the labellum have slight peaks in the visible blue-green (400–550 nm). All portions of the flower had more pronounced peaks in the red part (600–700 nm) of the visual spectrum. Diagram of visible spectrum was modified and used by permission from V. Blacus under Creative Commons Attribution-Share Alike 3.0 unported license. (b) Fabricated UV reflective, and UV florescent labella, used in the field trials. © B. A. Roy. (c) To determine whether reflectance of UV was important for landings, three different kinds of artificial labella, UV-reflective, UV-fluorescent, and white, were inserted into color-matched artificial flowers and compared to a true flower (positive) control. n = 18 × 30 min time blocks (9 h total). UV reflectance alone did not influence landings. While there was a treatment effect, flies visited the true flowers more often, and UV-reflective, UV-fluorescent, and white labella all received indistinguishable landing rates ($F_{3,127}$=14.15, P < 0.0001). Columns not sharing the same letter are significantly different. Error bars represent ± 1 SE.

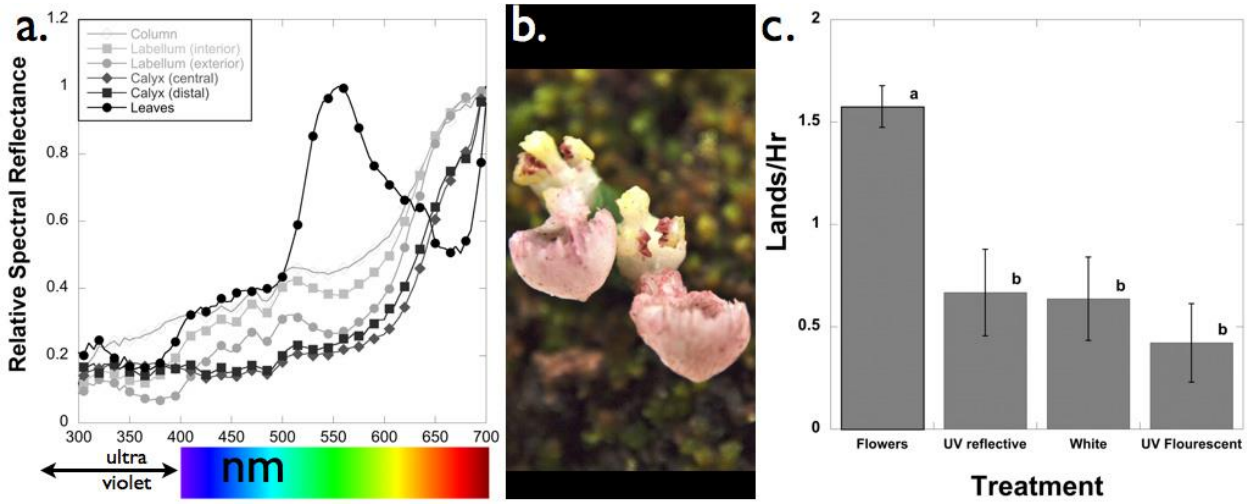


Fig. S4 Measuring contrasting patterns in *Dracula lafleurii*. Flowers were photographed in the field and then analyzed with IMAGEJ (http://rsb.info.nih.gov). To determine the proportions of red, green and blue (RGB) in each flower image we used the color profiler in ImageJ after first outlining the flower using the drawing tool. To determine variation in contrast, the images were split into RGB colors, and the red was used for analysis. The light color threshold for the red image was set to 185, the measurement scale was calibrated with actual measurements, and the overall area vs. area of light color of each flower was determined. This specimen was 18.1% light colored, or 81.9% pigmented. Pigmentation in sepals ranged from 38 to 93% (n = 22 flowers). Photos © B. A. Roy.

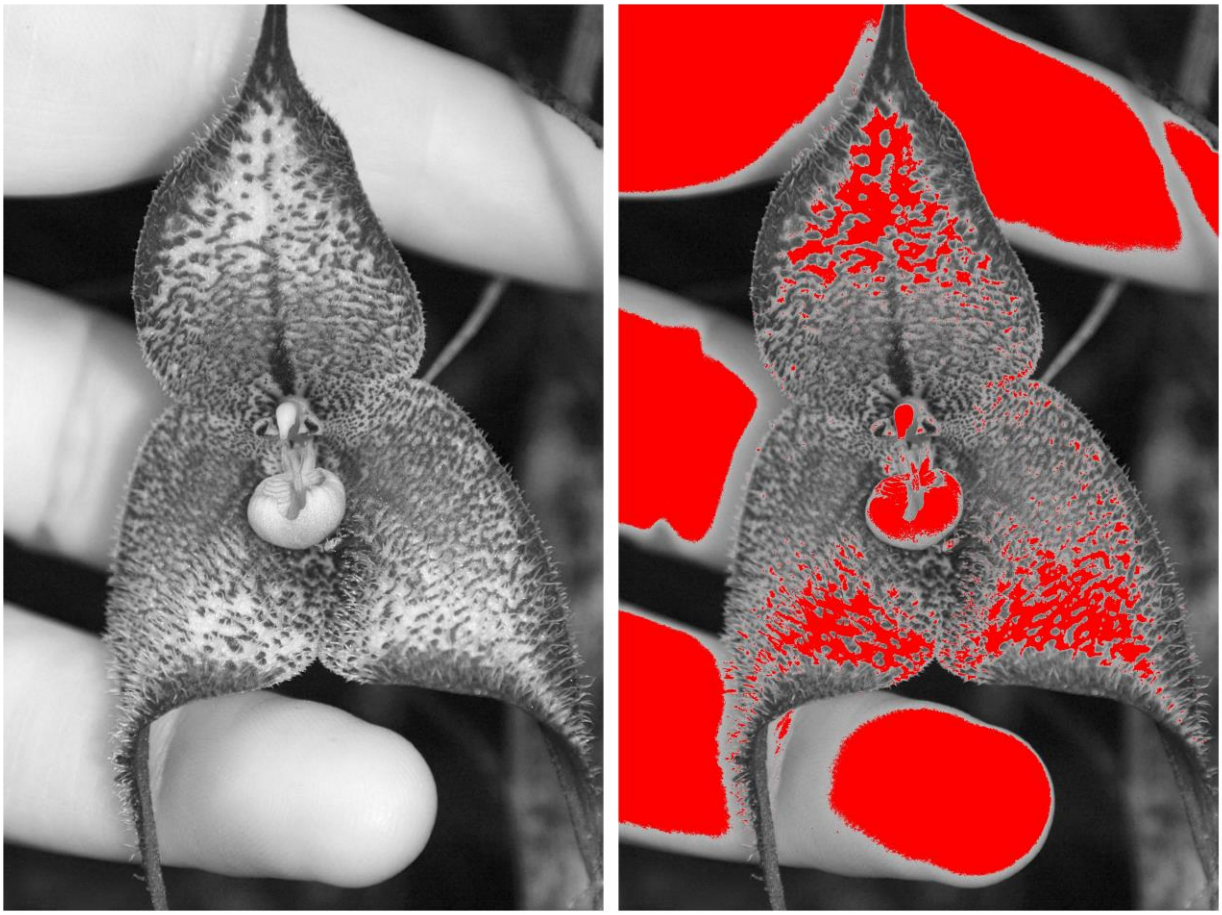


Fig. S5 (a, b) Mushroom specimens included in the volatile analysis. Images correspond to data in Table S2. (a) *Crepidotus*, *Entoloma*, *Junghuhnia* and *Mycena* photos © M. Wherley. Mycenaceae, *Pachylepyrium nubicola*, *Podoscypha* cf *venustula*, (b) *Crepidotus*, *Filoboletus gracilis*, *Pleurotopsis*, *Leucocoprinus* and *Polystictus* photos © J. McAlpine. *Psathyrella* and *Rigidoporus*, photos © T. Policha. All specimens deposited at Kew Royal Botanical Gardens (K) and the Herbario Nacional de Ecuador (QCNE).

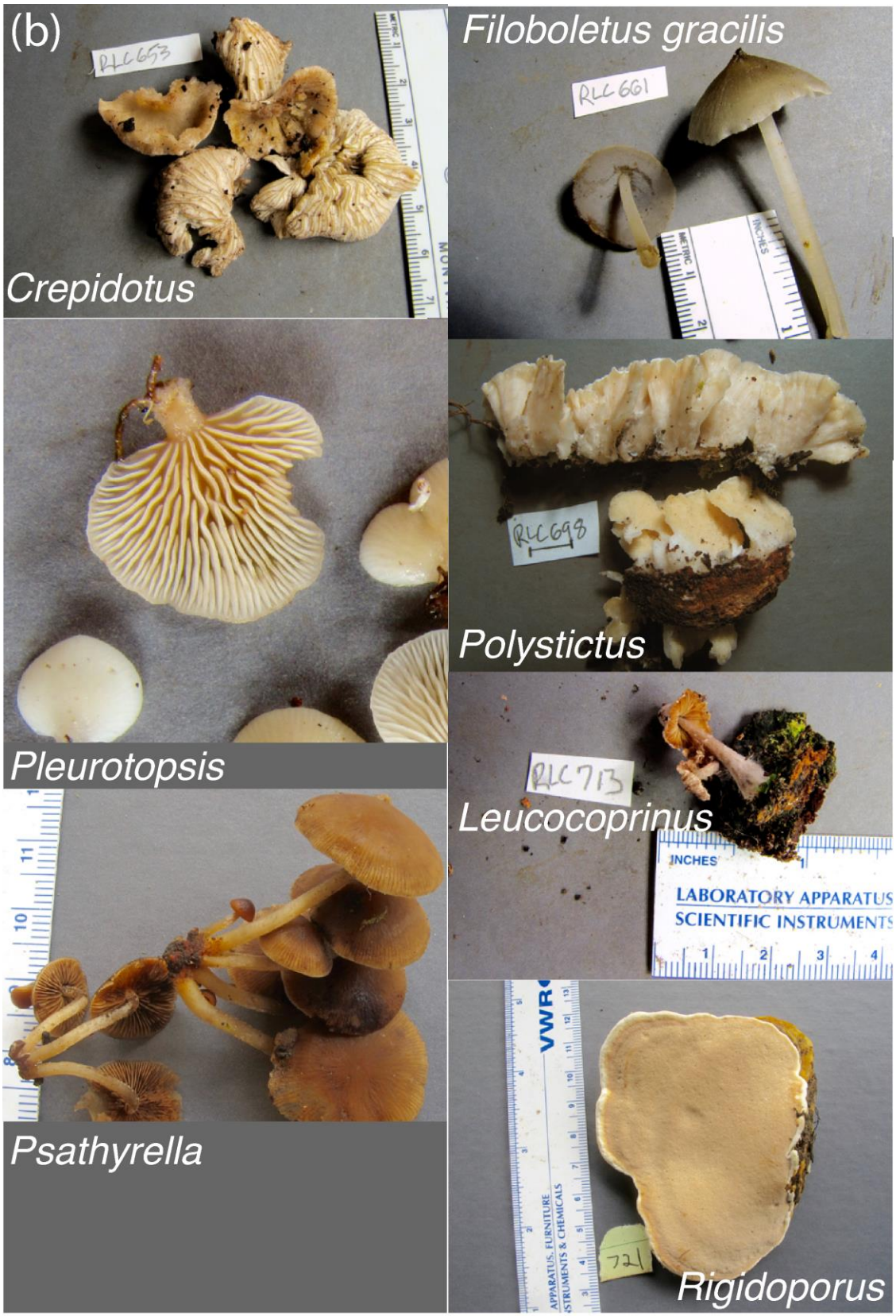

(b)
Crepidotus
Filoboletus gracilis
Polystictus
Pleurotopsis
Leucocoprinus
Psathyrella
Rigidoporus

Fig. S6 Pleurothallid orchid species included in the volatile analysis. All photos taken at Reserva Los Cedros. *Brachonidium n* = 1; *Masdevallia nidifica n* = 6; *Masdevallia ximena n* = 7; *Poroglossum* cf *hoeijeri n* = 1; *Scaphosepalum n* = 3; *Trisetella dalstroemii n* = 1. All photos from Policha (2012) and used with permission. **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.

Fig. S7 Landing location preference depends on treatment in real, artificial and chimeric flowers. Goodness-of-fit G-tests show deviation from the null hypothesis of random landings in all cases, but the preferred region depends on the treatment. (a) Real Flower: regions closest to the column (ventral calyx, and labellum, exterior and interior) were preferentially approached G=106.05 df 4, $P < 0.0001$. (b) Real Labellum Chimera: a similar pattern was seen; points closest to the column were preferentially approached. G=45.49 df 4, $P < 0.0001$ (c) Real Calyx Chimera: showed strong preference for the various parts of the calyx, with the labellum being largely avoided. G=38.03 df 4, $P < 0.0001$ (d) Artificial Flower: landings to this treatment were exclusively to the calyx, and particularly to regions most distal to the column, (the dorsal side and the tails). G=37.41 df 4, $P < 0.0001$.

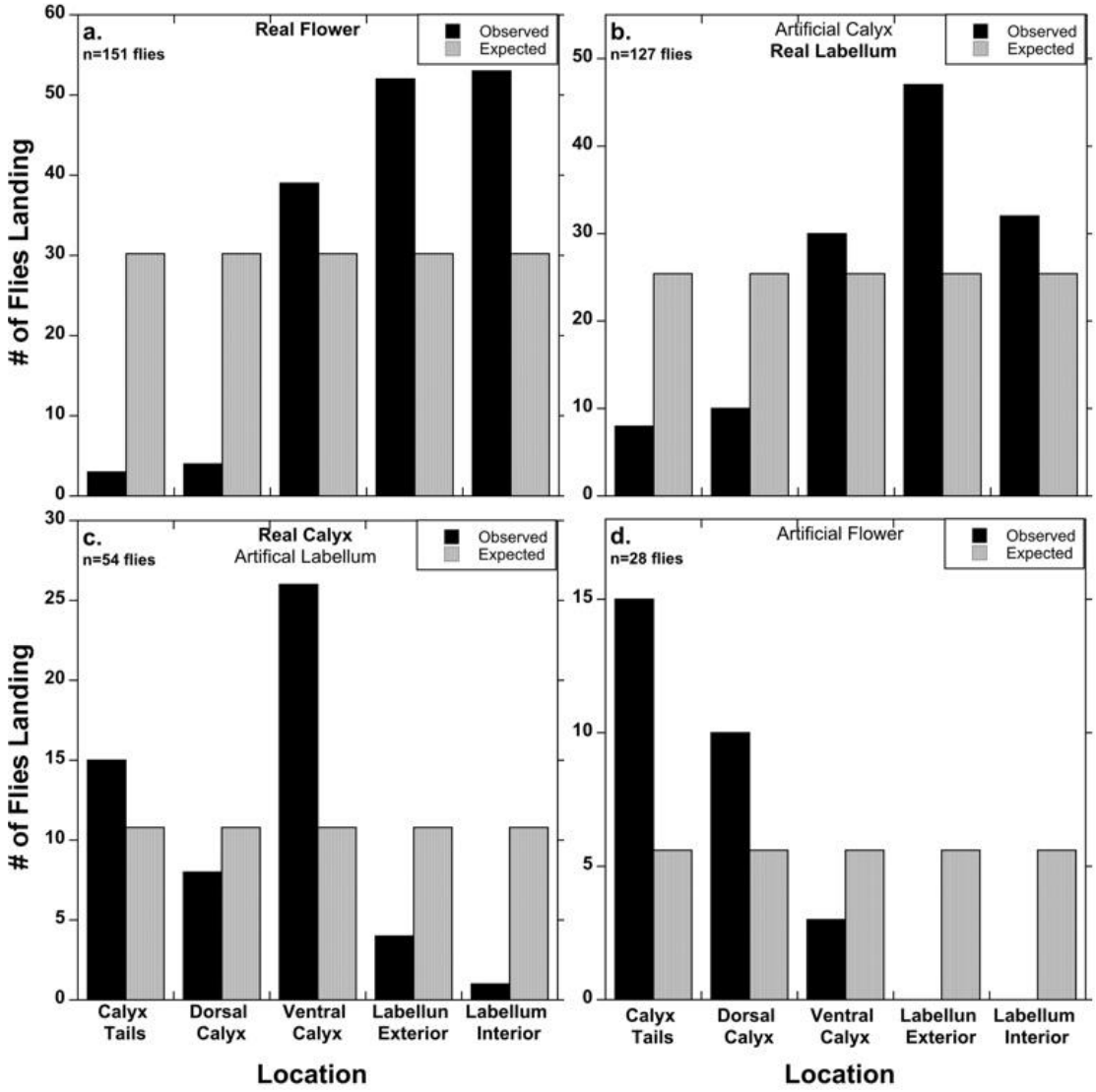


Fig. S8 *Dracula vampira* (Luer) Luer. This greenhouse grown specimen illustrates the calyx patterns of this Ecuadorean endemic. Marsh Hollow Orchids, Fenwick, Ontario. Photo © T. Policha.

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

Hypothesis	**Experiment**	**Response variable**	**Contrast**	**Result (*P*<0.05 in bold)**	**Sample size (visitors to whole array)**	**Duration***	**Dates**
Visual signals are sufficient for attraction	Deconstruction	Approaches/Hr & Lands/Hr	"Visual Only" vs. "Visual Control"	Approaches: $t_{1,9.26}$=2.59; ***P*=0.014.** Lands: $t_{1,11.38}$=1.13; *P*=0.142.	Approaches: n=420 Lands: n=130	10 replicates = 5 hours	Jan. 31-Feb. 10, 2010
H_0: Visual signals are as attractive as a real flower	Deconstruction	Approaches/Hr & Lands/Hr	"Visual Only" vs. Real Flower	Approaches: $t_{1,9.13}$=-1.86; ***P* =0.048.** Lands: $t_{1,9.02}$=-2.07; ***P* =0.034**.	Approaches: n=420 Lands: n=130	10 replicates = 5 hours	Jan. 31-Feb. 10, 2010
Olfactory signals are sufficient for attraction	Deconstruction	Approaches/Hr & Lands/Hr	"Scent Only" vs. "Scent Control"	Approaches: $t_{1,16.64}$=-1.96; ***P*=0.033.** Lands: $t_{1,17.96}$=-0.474; *P*=0.321.	Approaches: n=420 Lands: n=130	10 replicates = 5 hours	Jan. 31-Feb. 10, 2010
H_0: Olfactory signals are as attractive as a real flower	Deconstruction	Approaches/Hr & Lands/Hr	"Scent Only" vs. Real Flower	Approaches: $t_{1,9.01}$=1.98; ***P* =0.040.** Lands: $t_{1,9.01}$=2.07; ***P* =0.034.**	Approaches: n=420 Lands: n=130	10 replicates = 5 hours	Jan. 31-Feb. 10, 2010
Visual signals are sufficient for attraction	Reconstruction	Approaches/Hr & Lands/Hr	"Visual Only" vs. "Material Control"	Approaches: $t_{1,25.7}$=2.72; ***P* =0.0105.** Lands: $t_{1,28.6}$=0.39; *P*=0.35	Approaches: n=99 Lands: n=61	18 replicates = 9 hours	Jan.15 -Mar.7, 2011
Scent increases	Reconstruction	Approaches/Hr &	"Visual+Scent" vs. ""Solvent	Approaches: $t_{1,25.3}$=2.79;	Approaches: n=99	18 replicates	Jan.15 -Mar.7,

attraction		Lands/Hr	Control"	***P* =0.005.** Lands: $t_{1,19.2}$ =2.49; ***P* =0.011**	Lands: n=61	= 9 hours	2011
We reconstructed the phenotype	Reconstruction	Approaches/Hr & Lands/Hr	"Visual+Scent" vs. Real Flower	Approaches: $t_{1,20.7}$ =1.43; *P*=0.084. Lands: $t_{1,18.4}$ =1.68; *P*=0.055	Approaches: n=99 Lands: n=61	18 replicates = 9 hours	Jan.15 -Mar.7, 2011
H_0: A real labellum without a real calyx is as attractive as a real flower	Chimeras	Lands/Hr	"Real Labellum Chimera" vs. Real Flower	$t_{1,23.4}$ =-0.357 *P*=0.362	n=372	13 replicates = 6.5 hours	Feb. 8-15, 2011
Visual signals only are not as attractive as a real flower	Chimeras	Lands/Hr	"Artificial Flower" vs. Real Flower	$t_{1,13.4}$ =-2.10 ***P*=0.0275**	n=372	13 replicates = 6.5 hours	Feb. 8-15, 2011
A real calyx without real labellum is not as attractive as a real flower	Chimeras	Lands/Hr	"Real Calyx Chimera" vs. Real Flower	$t_{1,13.4}$ =-2.10 ***P* =0.0275**	n=372	13 replicates = 6.5 hours	Feb. 8-15, 2011
H_0: Spotted artificial (unscented) flowers are as attractive as real flowers	Visual Signals (pattern/contrast)	Lands/Hr & Visit duration	"Spotted" vs. Real Flower	Lands: $t_{1,25}$ =-1.50 *P*=0.1805 Duration: $t_{1,5.1}$ =2.22; ***P*=0.038**	n= 44	6 replicates = 3 hours	Jan.22 - Feb.15, 2011
H_0: Striped artificial (unscented) flowers are as attractive as real flowers	Visual Signals (pattern/contrast)	Lands/Hr & Visit duration	"Striped" vs. Real Flower	Lands: $t_{1,25}$ =2.60 ***P*=0.008** Duration: $t_{1,5.1}$ =2.38; ***P*=0.031**	n= 44	6 replicates = 3 hours	Jan.22 - Feb.15, 2011
H_0: White artificial (unscented)	Visual Signals (color)	Lands/Hr & Visit duration	"White" vs. Real Flower	Lands: $t_{1,25}$ =2.87 ***P*= 0.049**	n= 44	6 replicates = 3 hours	Jan.22 - Feb.15,

flowers are as attractive as real flowers				Duration: $t_{1,5}$ =2.48 ***P*= 0.028**			2011
H_0: Red artificial (unscented) flowers are as attractive as real flowers	Visual Signals (color)	Lands/Hr & Visit duration	"Red" vs. Real Flower	Lands: $t_{1,25}$ =3.14 ***P*=0.038** Duration: $t_{1,5}$ =2.50 ***P*=0.0275**	n= 44	6 replicates = 3 hours	Jan.22 - Feb.15, 2011

Note: *, each 30 min observation period was considered a replicate.

Table S2 The 15 most common volatiles found (ordered by retention time), including where they were found and the number of samples containing them

	RT	Kovats	*Dracula lafluerii* Calyces	*Dracula lafluerii* Petals	*Dracula lafluerii* Labella	*Dracula lafluerii* Columns	Mushrooms	Pleurothallids
3-methyl-1-butanol	8.66	1222					1	1
3-octanone	9.42	1260					10	
3-octanol	11.5	1394					2	
cis-furanoid linalool oxide	12.25	1446						8
trans-furanoid linalool oxide	12.64	1473						7
1-octen-3-ol	12.27	1447			15	1	16	
benzaldehyde	13.27	1527	1		1		11	1
linalool	13.62	1545						7
cis-pyranoid linalool oxide	16.08	1722						7
trans-pyranoid linalool oxide	16.34	1752						7
methyl salicylate	16.42	1782	9			15		6
benzyl alcohol	17.72	1882	4	4	20	8		9
2-phenylethanol	18.11	1917		2	8		8	9
eugenol	20.75	2178						6
isoeugenol	22.47	2363						1

Notes: The retention times on a polar ethylene glycol capillary column (EC™ Wax; W. R. Grace & Co. Columbia, Maryland) (30 m × 0.25 mm i.d., film thickness 0.25 μm), Kovats indices, and the number of samples in each group containing the compounds listed. In this study we analyzed dissected *Dracula* flowers (n = 23), co-ocurring mushrooms (n = 32), and nonmushroom-mimicking pleurothallid orchids (n = 30). All compound identifications were verified using authentic standard compounds.

Table S4 Mushroom specimens collected for scent analysis

Family	***Genus***	***species***	**Collection # (RLC)**	**Collector**	**Date (mm/dd/yyyy)**
Crepidotaceae	*Crepidotus*		404	R. Manobanda	01/22/2010
Marasmiaceae	*Entoloma*		407	R. Manobanda	01/22/2010
Strophariaceae	*Gymnopilus*		408*	B. Roy	01/24/2010
Polyporaceae	*Junghuhnia*		409*&410	B. Roy, R. Manobanda	01/24/2010
Mycenaceae	*Mycena*		411	J. McAlpine	01/24/2010
Polyporaceae	*Ganoderma*	*australe*	413*	A. Troya	01/25/2010
Mycenaceae			630	F. Bechtolsheim	02/01/2010
Strophariaceae	*Pachylepyrium*	*nubicola*	637	A. Troya	02/02/2010
Meruliaceae	*Podoscypha*	*cf. venustula*	640	A. Troya	02/02/2010
Crepidotaceae	*Crepidotus*		653	F. Bechtolsheim & J. McAlpine	02/03/2010
Mycenaceae	*Filoboletus*	*gracilis*	661	T. Policha	02/04/2010
Tricholomataceae	*Pleurotopsis*		668	J. McAlpine	02/09/2010
Polyporaceae	*Polystictus*	*membranaceus*	698	J. McAlpine, B. Roy	02/12/2010
Agaricaceae	*Leucocoprinus*		713	F. Bechtolsheim	02/14/2010
Psathyrellaceae	*Psathyrella*		719	R. Manobanda	03/20/2010
Meripilaceae	*Rigidoporus*		721	R. Manobandna	03/20/2010
Mycenaceae	*Filoboletus*	*gracilis*	no data*	no data	02/11/2010

Note: *, no photo

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

Additional chromatographic analyses were performed in order to identify which enantiomer of 1-octen-3-ol, the volatile compound putatively responsible for fungal mimicry in *Dracula lafleurii* orchids, is produced by the labellum. Both the S-(+) and R-(-) enantiomers of this compound are present in nature, with the R-(-) form of 1-octen-3-ol (so-called 'mushroom oil') known as a dominant component of fungal fruiting bodies (Zawirska-Wojtasiak, 2004). A chiral GC column (Cyclosil-B, Agilent Technologies, Inc.; 30 m long, 0.25 mm inner diameter, 0.25 µm film thickness) was used with a splitless injection at 240°C, column temperature increase from 40 to 240°C at 10°C min^{-1}. Under these conditions, the S-(+) and R-(-) enantiomers can be distinguished by co-injection of an authentic standard (product number O0159, TCI America, Inc.), along with a 1 h extract of commercial mushroom cap tissue (*Agaricus bisporus*) in hexane, which has been shown to be dominated (>98%) by the R-(-) enantiomer (Zawirska-Wojtasiak, 2004). Several replicate extracts of labella from *D. lafleurii*, along with those of related species (*D. morleyi, D. pubescens*) and co-occurring mushrooms were analyzed in this way.

Results: Chiral GC-MS analyses unambiguously identified R-(-)-1-octen-3-ol as the exclusive enantiomer present in the solvent extracts of *Dracula* orchid labella as well as the caps of co-occurring fungal fruiting bodies, given the co-elution of that peak with authentic standards and the dominant peak of the commercial mushroom extract run as positive controls. These results confirm that the putative chemical mimicry of mushroom scent by *Dracula* orchid labella has chiral specificity.

Reference

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.