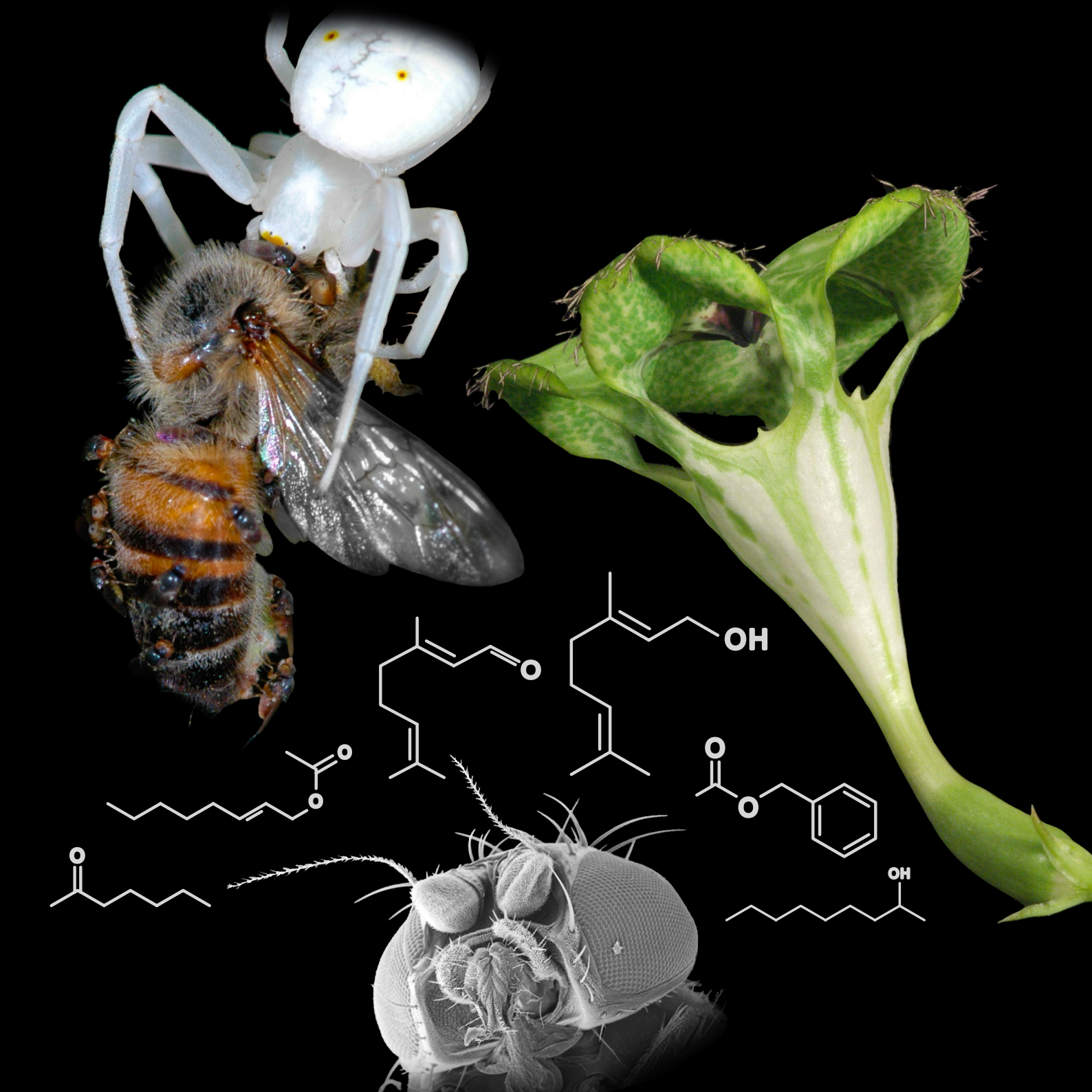
Chemical Ecology of Pollination in Deceptive *Ceropegia*



CHEMICAL ECOLOGY OF POLLINATION IN DECEPTIVE *CEROPEGIA*

DISSERTATION
zur Erlangung des Doktorgrades
Dr. rer. nat.

an der Bayreuther Graduiertenschule für Mathematik und Naturwissenschaften (BayNAT) der Universität Bayreuth

vorgelegt von
Annemarie Heiduk

Bayreuth, Januar 2017

Die vorliegende Arbeit wurde in der Zeit von Februar 2012 bis Dezember 2016 in Bayreuth am Lehrstuhl Pflanzensystematik unter der Betreuung von Herrn Univ.-Prof. Dr. Stefan Dötterl (Erst-Mentor) und Herrn PD Dr. Ulrich Meve (Zweit-Mentor) angefertigt.

Gefördert wurde die Arbeit von Februar bis April 2012 durch den 'Feuerwehrfond' zur Doktorandenförderung der Universität Bayreuth, von Mai 2012 bis April 2015 durch ein Stipendium nach dem Bayerischen Eliteförderungsgesetzt (BayEFG), und von Mai bis Juli 2015 durch ein Stipendium des Bayerischen Programms zur Förderung der Chancengleichheit für Frauen in Forschung und Lehre.

Vollständiger Abdruck der von der Bayreuther Graduiertenschule für Mathematik und Naturwissenschaften (BayNAT) der Universität Bayreuth genehmigten Dissertation zur Erlangung des akademischen Grades eines Doktors der Naturwissenschaften (Dr. rer. nat.).

Dissertation eingereicht am: 02.02.2017

Zulassung durch das Leitungsgremium: 10.02.2017

Wissenschaftliches Kolloquium: 31.05.2017

Amtierender Direktor: Prof. Dr. Stephan Kümmel

Prüfungsausschuss:

Prof. Dr. Stefan Dötterl	(Erstgutachter)
Prof. Dr. Konrad Dettner	(Zweitgutachter)
Prof. Dr. Heike Feldhaar	(Vorsitz)
Prof. Dr. Bettina Engelbrecht	

Declaration of self-contribution

This dissertation is submitted as a “Cumulative Thesis“ and contains a general synopsis (Part I) and three manuscripts (Part II) about the chemical ecology and pollination biology of *Ceropegia*. The major part of the research presented here was accomplished by myself under supervision of Univ.-Prof. Dr. Stefan Dötterl (Universities of Bayreuth and Salzburg) and PD Dr. Ulrich Meve (University of Bayreuth).

In collaboration with my supervisors, I developed the methods, collected most of the data, and drafted the manuscripts. All co-authors discussed the results and supported preparation of manuscripts.

1st manuscript:

Authors: Annemarie Heiduk, Hanghui Kong, Irina Brake, Michael von Tschirnhaus, Till Tolasch, Armin G. Tröger, Elisabeth Wittenberg, Wittko Francke, Ulrich Meve, and Stefan Dötterl

Title: Deceptive *Ceropegia dolichophylla* fools its kleptoparasitic fly pollinators with exceptional floral scent

Status: Published on 03 July 2015 in *Frontiers of Ecology and Evolution* 3: 66. doi: 10.3389/fevo.2015.00066

Own contribution: concept and study design 60%, data acquisition 70%, analyses of scent samples 95%, data analyses 90%, preparation of figures and tables 100%, discussion of results 70%, manuscript writing 90%.

The study was designed by AH, SD, and UM. Bioassays in China in 2012 were performed by HK. AH collected all other field data. Electrophysiological measurements were performed by SD. Identification of pollinators was carried out by IB and MV. Identification and/or synthesis of some of the compounds were done by WF, AT, EW, and TT. AH analyzed the data and wrote the first draft of the manuscript. WF wrote the part on the chemical synthesis. All authors contributed to interpretation of the findings, and edited and approved the manuscript.

2nd manuscript:

Authors: Annemarie Heiduk, Irina Brake, Michael von Tschirnhaus, Matthias Göhl, Andreas Jürgens, Steven D. Johnson, Ulrich Meve, and Stefan Dötterl

Title: *Ceropegia sandersonii* mimics attacked honeybees to attract kleptoparasitic flies for pollination

Status: Published on 24 October 2016 in *Current Biology* 26, 2787-2793.

Own contribution: concept and study design 50%, data acquisition 70%, analyses of scent samples 90%, data analyses 90%, preparation of figures and tables 95%, discussion of results 70%, manuscript writing 85%.

The study was conceived by AH, SD, and UM. The experiments were designed by AH, AJ, and SD. Data were collected by AH, AJ, SD, and UM. Statistical analyses were performed by AH, SDJ, and SD. Flies were identified by IB and MvT. (*E*)-2-octen-1-yl acetate was synthesized by MG. Lab facilities and equipment at UKZN, South Africa was provided by SDJ. The manuscript was drafted by AH and all authors contributed valuable discussions.

3rd manuscript:

Authors: Annemarie Heiduk, Irina Brake, Michael von Tschirnhaus, Jean-Paul Haenni, Raymond Miller, John Hash, Samuel Prieto-Benítez, Andreas Jürgens, Steven D. Johnson, Stefan Schulz, Sigrid Liede-Schumann, Ulrich Meve, and Stefan Dötterl

Title: Floral scent and pollinators of *Ceropegia* trap flowers

Status: Published on 01 July 2017 in *Flora* 232, 169-182 (invited contribution).

Own contribution: concept and study design 60%, data acquisition 70%, analyses of scent samples and data 90%, preparations of figures and tables 100%, discussion of results 80%, manuscript writing 95%.

The study was conceived by AH, SD, and UM. Data were collected by AH, UM, AJ. Genetic analyses were performed by SLS and PBS. Statistical analyses were performed by AH. Flies were identified by IB, MvT, JPH, RM, and JH. Floral compounds of *C. stenantha* were identified and synthesized by SS. Lab facilities and equipment at UKZN, South Africa was provided by SDJ. The manuscript was drafted by AH and all authors contributed valuable discussions.

CONTENT

Abstract

Deceptive plants evolved fascinating traits to earn reproductive success without providing a reward. Most sophisticated are trap flowers, which evolved several times independently in angiosperms. They often have a complex and striking morphology, and floral scent is assumed to attract the pollinators from distance. However, in many systems, knowledge on pollinators, natural reproductive success, and the role of floral scent or specific components thereof for pollinator attraction is scarce.

One of the most species-rich genera with deceptive trap flowers is *Ceropegia* L. (Apocynaceae, Asclepiadoideae). It covers more than 200 species, mainly distributed in Old World (sub)tropical habitats, and all species studied so far are pollinated by Diptera, such as Ceratopogonidae, Chloropidae, and Milichiidae. Floral scents are believed to advertise non-existing rewards, such as food, oviposition sites, or sex pheromones. However, floral scent composition and mimicry strategies of *Ceropegia* are virtually unexplored (but see Heiduk et al., 2010).

I determined flower visiting/pollinating flies of 13 South African and one Chinese *Ceropegia* species, analyzed their floral scent composition using dynamic headspace and gas chromatography coupled to mass spectrometry (GC/MS), and in eight species thereof also assessed reproductive success. I tested for relationships between floral scent and visitor/pollinator patterns. Based on genetic similarities of the plants, I also tested for phylogenetic signals in scent chemistry and pollinators. In *C. sandersonii* and *C. dolichophylla* I identified scent components responsible for pollinator attraction by gas chromatography-electroantennographic detection (GC/EAD) and behavioral studies. This approach revealed the (potential) models mimicked by the flowers.

There was varying abundance of flies inside flowers of *Ceropegia*, and reproductive success was generally low. Flowers of studied species were mostly visited by only one or two pollinating fly families or genera, whereas pollinators overall belonged to eight families, 18 genera, and 33 morphospecies. Despite some overlap in flower visiting/pollinating taxa, the dominant pollinating morphospecies were species specific. There was phylogenetic signal in flower visiting fly families and in pollinator assemblages (family and morphospecies level).

Analysis of floral scent revealed high variability in quality and (semi-)quantity among studied species. Over 300 different volatiles were detected, mainly aliphatic and aromatic components, terpenoids, various unknowns, and new natural

components. Scent bouquets of studied species were unique, and many components, including main components (e.g., acetoin, 2-nonanol, (2*S*,6*R*,8*S*)-8-methyl-2-propyl-1,7-dioxaspiro[5.5]undecane) were species specific. There was no phylogenetic signal in floral scent chemistry, and neither flower visitor nor pollinator patterns correlated with floral chemistry.

Ceropegia sandersonii was mainly pollinated by kleptoparasitic *Desmometopa* flies (Milichiidae). These flies can frequently be found on honeybees being eaten by spiders, where they feed on secretions that drip from the bee. Honeybees under attack try to bite and sting the assailant and thereby release volatiles. My studies showed that i) distressed honeybees are highly attractive for *Desmometopa*, ii) the scent bouquets of *C. sandersonii* flowers and distressed honeybees have many components in common, and iii) some components thereof are responsible for attraction of *Desmometopa* to both distressed bees and to *C. sandersonii* flowers. I concluded that floral scent of *C. sandersonii* resembles an alarming honeybee to attract its pollinators, and that the plant has a kleptomyiophilous pollination strategy.

Flowers of *Ceropegia dolichophylla* were also pollinated by *Desmometopa* flies, and released volatiles uncommon among floral scents, especially spiroacetals and components, such as N-(3-methylbutyl)acetamide and 6-tridecene. However, several of the components, e.g., (*E*,*E*)-2,8-dimethyl-1,7-dioxaspiro[5.5]undecane and N-(3-methylbutyl)acetamide, are known venom constituents of predatory *Polistes* wasps and occur in cephalic secretions of *Andrena* bees. I hypothesized that *C. dolichophylla* also has a kleptomyiophilous pollination strategy and deceives its pollinators by mimicking volatiles of *Andrena* bees or *Polistes* wasps. Comparison of volatiles emitted by other study species points out that besides kleptomyiophily also other deceptive strategies, i.e., oviposition site mimicry, occur within *Ceropegia*.

My study contributes to understand the pollination biology and scent chemistry in *Ceropegia*, and chemical communication between deceptive plants and their pollinators. The finding of novel natural components in some species calls for further studies to identify their function in the biology of the deceived flies, and to reveal their importance in the deceptive system of *Ceropegia*.

Zusammenfassung

Im Laufe der Evolution haben Täuschblumen faszinierende Eigenschaften erworben, um ihren Reproduktionserfolg ohne Gegenleistung für Bestäuber zu sichern. Am raffiniertesten sind Fallenblüten, welche sich innerhalb der Angiospermen mehrmals unabhängig voneinander entwickelten. Man nimmt an, dass ein Großteil davon ihre Bestäuber maßgeblich durch Blütenduft anlockt. Für die meisten Bestäubungssysteme allerdings ist das Wissen über Bestäuber, Reproduktionserfolg und die Rolle des Blütenduftes bzw. spezifischer Duftstoffe zur Bestäuberanlockung spärlich.

Eine der artenreichsten Gattungen mit trügerischen Fallenblüten ist *Ceropegia* L. (Apocynaceae, Asclepiadoideae) mit über 200 Arten in (sub)tropischen Habitaten der Alten Welt. Soweit bekannt, wird *Ceropegia* von Dipteren, wie Ceratopogonidae, Chloropidae und Milichiidae bestäubt. Man vermutet, dass die Blütendüfte Futter, Eiablageplätze oder Sexualpheromone imitieren. Jedoch sind die Duftstoffe und Mimikry-Systeme von *Ceropegia* nahezu unerforscht (außer Heiduk et al., 2010).

Im Rahmen meiner Doktorarbeit untersuchte ich 14 *Ceropegia*-Arten (13 südafrikanische, eine chinesische). Ich erhob Daten zum Reproduktionserfolg, bestimmte blütenbesuchende/bestäubende Fliegen, analysierte die Blütendüfte mittels gekoppelter Gaschromatographie/Massenspektroskopie, und testete, ob ein Zusammenhang zwischen Blütenduft und Blütenbesuchern/Bestäubern besteht. Basierend auf genetischen Ähnlichkeiten der untersuchten Arten prüfte ich, ob es ein phylogenetisches Signal in der Blütenduftchemie bzw. den Blütenbesucher- und Bestäubermustern gibt. Für *C. sandersonii* und *C. dolichophylla* identifizierte ich mittels elektroantennographischer Messungen und Verhaltensexperimenten jene Duftstoffe, die für die Bestäuberanlockung verantwortlich sind.

Bei den untersuchten *Ceropegia*-Arten war der Reproduktionserfolg im Allgemeinen gering. Die Bestäuber gehörten insgesamt zu acht Familien, 18 Gattungen und 33 Morphotypen. Generell wurden die Blüten der verschiedenen Arten von ein oder zwei Fliegenfamilien oder -gattungen besucht, wobei die dominierenden Bestäubergruppen meist artspezifisch waren. Ein phylogenetisches Signal zeigte sich in den blütenbesuchenden Fliegenfamilien und in der Bestäuberzusammensetzung (Familien- und Morphotyp-Ebene). Die Blütenduftanalysen ergaben, dass der Duft sowohl (semi-)quantitativ als auch qualitativ zwischenartlich hoch variabel ist.

Insgesamt fand ich über 300 Duftstoffe, hauptsächlich aliphatische Verbindungen, aromatische Stoffe, Terpenoide sowie viele unbekannte Substanzen und auch neue Naturstoffe. Jede *Ceropegia*-Art hatte ein eigenes Duftbouquet und viele Komponenten, inklusive der Hauptkomponenten (z.B., Acetoin, 2-Nonanol, (2*S*,6*R*,8*S*)-8-Methyl-2-propyl-1,7-dioxaspiro[5.5]undecan), waren artspezifisch. Es gab kein phylogenetisches Signal in der Blütenduftchemie, und weder Blütenbesucher noch Bestäuber korrelierten mit dem Blütenduft.

Blüten von *Ceropegia sandersonii* werden hauptsächlich von kleptoparasitischen *Desmometopa* Fliegen (Milichiidae) bestäubt. Diese Fliegen findet man häufig auf frisch durch Spinnen erbeuteten Honigbienen. Sie saugen dort Sekrete auf, die aus dem Bienenkörper austreten. Eine angegriffene Biene versucht ihren Angreifer zu stechen und zu beißen. Dabei gibt sie Duftstoffe ab. Ich konnte zeigen, dass i) angegriffene Honigbienen hoch attraktiv für *Desmometopa*-Fliegen sind, ii) der Blütenduft viele Duftstoffe enthält, die auch angegriffene Bienen abgeben, und iii) einige dieser Duftstoffe für die Anlockung von *Desmometopa* zu sich wehrenden Bienen bzw. zu den Blüten von *C. sandersonii* verantwortlich sind. Daraus folgerte ich, dass der Blütenduft für die bestäubenden Fliegen eine Honigbiene in Not darstellt und diese Pflanze somit eine kleptomyiophile Bestäubungsstrategie hat.

Die Blüten von *Ceropegia dolichophylla*, die ebenfalls von *Desmometopa* bestäubt werden, gaben für Blütendüfte sehr ungewöhnliche Substanzen ab, insbesondere Spiroacetale, und Stoffe wie N-(3-Methylbutyl)acetamid und 6-Tridecan. Etliche dieser Duftstoffe, z.B. (*E*,*E*)-2,8-Dimethyl-1,7-dioxaspiro[5.5]undecane und N-(3-Methylbutyl)acetamid, sind aus dem Gift räuberischer *Polistes*-Wespen bzw. aus Kopfdrüsensekreten von *Andrena*-Bienen bekannt. Man kann davon ausgehen, dass *C. dolichophylla* ebenfalls kleptomyiophil ist und Bestäuber betrügt, indem sie *Polistes*-/*Andrena*-Duft nachahmt. Ein Vergleich der bei den anderen Arten gefundenen Substanzen mit Literaturdaten weist darauf hin, dass in *Ceropegia* neben Kleptomyiophilie auch andere Betrugsstrategien, wie etwa Brutsubstrat-Mimikry, vorkommen.

Meine Forschungsarbeit trug maßgeblich dazu bei, die Bestäubungsbiologie und Blütenduftchemie von *Ceropegia*, sowie generell die chemische Kommunikation zwischen Täuschblumen und ihren Bestäubern besser zu verstehen. Die Entdeckung neuer Naturstoffe verlangt nach weiteren Untersuchungen, um deren Funktion in der

Biologie der betrogenen Fliegen sowie bei der Bestäubung von *Ceropegia* aufzuklären.

PART I - Synopsis

General introduction

Pollination in general

According to latest approximations, there are nearly 400000 described species of angiosperms (Lughadha et al., 2016; Pimm and Joppa, 2015). Flowering plants bear enormous diversity and species-richness in terrestrial as well as aquatic habitats (Bell et al., 2010). Though animal pollination was established before the rise of angiosperms, the evolutionary driving force for their dazzling diversification probably was the shift from mere abiotic (wind, water) to multiple possible ways of biotic (animals) pollination, and the subsequent adaptation of flowers to animal pollinators (Baker and Hurd Jr., 1968; Kevan and Baker, 1983; Willmer, 2011). The vast majority of flowering plants depends on animals for reproduction (Ollerton et al., 2011), and insects are by far the most important pollinators (Grimaldi, 1999; Shivanna, 2014).

Flowers are morphologically complex constructs in which floral features such as color, shape, nectar, and scent work alone or in combination to attract and manipulate pollinators in favor of the plants' sexual reproduction (Willmer, 2011). Visual cues together with flower scents play major roles in pollinator attraction (e.g., Raguso and Willis, 2002; Wright and Schiestl, 2009; see also Dudareva and Pichersky, 2006, and references therein) and can effect species isolation (e.g., Amrad et al., 2016; Byers et al., 2014; Sheehan et al., 2016; Vickery, 1995), whereas only scent bouquets are mostly unique for each species (Dobson, 1994; Willmer, 2011). Floral scents are intricate combinations of chemical volatiles, not seldom exceeding a hundred of components (Raguso, 2008). The identity and number of different scent components as well as the amount contributed by each component show high inter- and intraspecific variability (Dudareva and Pichersky, 2000; Knudsen and Gershenzon, 2006). Emission of volatiles can be flexible timewise and/or vary among different organs (Dötterl and Jürgens, 2005; Martin et al., 2016). It has been recognized long ago that floral scents differ among plants, and that floral volatiles are associated with pollinator attraction (Delpino, 1873; Kerner von Marilaun, 1895). However, only modern techniques allow detailed analyses of floral scents and their relevance for pollinator attraction. Suitable methods to determine qualitative and quantitative dimensions of floral scents are dynamic headspace sampling followed by gas chromatography coupled to mass spectrometry (GC/MS) (Dudareva and Pichersky, 2000).

The presence of a certain scent component not necessarily confirms its functionality for pollinator attraction because floral volatiles also mediate several other interactions, e.g., deterrence of facultative and antagonistic visitors such as florivores (Junker et al., 2010) or nectar thieves (e.g., Junker and Blüthgen, 2008). Insects differ quantitatively and/or qualitatively in perception of and responses to volatiles present in their environment (de Bruyne and Baker, 2008; Galizia and Menzel, 2000). To determine whether or not a scent component contributes to pollinator attraction, further analyses, i.e., electrophysiological and behavioral studies are needed. In plant-pollinator interactions gas chromatography coupled to electroantennographic detection (GC/EAD) is an elegant tool to identify volatile components that can be detected by the antennae of insects (Schiestl and Marion-Poll, 2002), and to study the role of floral scent and specific scent components in pollinator attraction. Whereas floral scents often function as long- and short-range attractant (Dudareva and Pichersky, 2000), morphological and color features function at short range and "guide" pollinators on the flower to fulfill pollination (e.g., Streinzer et al., 2009). Several plant species, though not being phylogenetically close, have similar suites of covarying morphological, visual, and olfactory features and share pollinators. A widely used term for such congruities of plants is "syndrome" (Faegri and van der Pijl, 1979; Quintero et al., 2016), and the conceptualization of this term dates back to the 19th century (Delpino, 1869; Knuth, 1898-1905; Müller, 1881). Under the premise that floral traits reflect an adaptation to a certain pollinator type, floral syndromes allow to assign plant species to pollination guilds, and to predict the pollinators (Ollerton and Watts, 2000; Waser et al., 1996). This classifying definition of pollination syndromes helps to categorize the overwhelming variety of plant-pollinator interactions.

Some plants are served by diverse pollinator assemblages, i.e., they are pollination generalists, others are specialists intimately associated with only few/single functional groups or even single species (e.g., *Yucca* spp.: see Pellmyr et al., 1996; *Ficus* spp.: see Rasplus, 1996). The extent of specialization of both interaction partners is not linked but often asymmetric, so that one partner might be highly specialized whereas the other one is a generalist (Armbruster et al., 2000; Vázquez and Simberloff, 2002). In generalized interactions, several unrelated and functionally redundant pollinators compete for resources, and may, due to different foraging strategies, have divergent selective pressures on the plant (e.g., Rodríguez-

Gironés and Santamaría, 2007), which itself competes for pollinators with coexisting plants (Geib and Galen, 2012; van der Kooi et al., 2016). This leads to much higher ecological complexity than in specialized systems where morphological and other features of flowers are adapted to very few and quite specific pollinators.

Mutualistic pollination and unidirectional exploitation

Regardless of whether generalist or specialist, the relationships that have evolved between flowering plants and pollinating animals can either be mutualistic or unidirectional. Mutualistic relations with benefit to both partners are either obligate or facultative. A famous text book example for a highly specialized obligate mutualistic interaction is *Ficus* and its pollinating agaonid wasps. In this system, both partners dependent on each other for reproduction and survival (West and Herre, 1994), and benefits/expenses are modified by partner abundance (Geib and Galen, 2012). Another example for obligate pollination mutualism is the relationship between oil-collecting bees and oil-producing flowers (e.g., Schäffler et al., 2015; Vogel, 1989). Facultative mutualistic pollination systems are based on compensability of both partners, i.e., there are multiple pollinators which themselves can visit several different plant species, whereby the species connectedness can form seriously intricate webs of interactions (Dupont et al., 2003). The basic requirement for mutual interactions is being honest. However, cheaters are ubiquitous, and there are numerous means and methods for plants to swindle (Dafni, 1984; Smithson, 2009) and for flower visitors to illegitimately obtain rewards (e.g., Castillo et al., 2013; Inouye, 1980, 2010; Irwin et al., 2010), whereby the severity of fitness consequences for the duped partner is broad-ranged. Plants can swindle by offering a different reward than they advertise or by not providing any reward at all (Renner, 2006). The expression “reward” not only refers to food for the flower visitor itself, such as nectar, pollen, and plant tissues, but also involves, among other rewards, nutrition for offspring, nesting material, mating place, warmth and hide out, and perfumes for courtship (Renner, 2006).

The term “unidirectional exploitation” (*sensu* Dafni, 1984) denotes all non-mutualistic pollination syndromes where non-rewarding plants exploit flower visitors and/or flower visitors commit floral larceny, i.e., theft and robbery of nectar or pollen (Dafni, 1984; Inouye, 1980). In the following, the attention is directed to systems,

where a non-rewarding plant misleads visitors by advertising a reward it does not provide, and is thus deceptive.

Among flowering plants, the proportion of deceptive species reaches 4% - 6% (Renner, 2006; Vogel, 1993). By whatever means the plants trick their visitors into non-rewarded pollination, the strategies must be beneficial to be evolutionary successful. Deceptive plants may for example gain better fitness when the resources which are not spent for reward production are invested for seed production, fruit set, and flower development (Jersáková et al., 2009; Thakar et al., 2003). Deceptive plants often exhibit low levels of fruit set (Tremblay et al., 2005), but under given circumstances they can have an evolutionary advantage in terms of elevated rates of cross-pollination when compared to rewarding species (Jersáková et al., 2006; Schiestl, 2005).

The deceptive strategies of plants involve both floral morphology (i.e., structural, tactile, and visual cues) and scent (i.e., olfactory cues) to exploit innate and/or learned responses of the target animal. In mimicry strategies, flowers should show high similarity to the model object to address the sensory abilities of the target animal and to trigger the same response as the mimicked model would (Galizia et al., 2005; Johnson et al., 2003; Renner, 2006). Though the inability of the flower visitor to recognize the fraud principally suffices for successful pollination (e.g., Johnson, 2000), additional traits, e.g., trapping devices (see below), may be required. Thus, flowers pollinated by deceit often have very complex floral structures, including trapping structures, in which pollinators are temporarily imprisoned (Ollerton et al., 2009, and references therein). The structures do not necessarily resemble the model mimicked by the flower, but may promote pollen removal and deposition as generally believed for adapted floral traits (Herrera, 1996; Proctor et al., 1996).

Trap flowers

Catching pollinators to ensure pollination is one of the most specialized and complex mechanism that plants have evolved for sexual reproduction. Detention of insect pollinators, either inside single flowers or inflorescences to ensure pollination has evolved several times independently in unrelated plant groups worldwide. A vast number of plants in different plant families, such as the Araceae, Aristolochiaceae, Apocynaceae-Asclepiadoideae, Hydnoraceae, Sterculiaceae, Burmanniaceae, and Annonaceae have evolved strategies and mechanisms to make pollinators stay

inside their (trap) flowers/blossoms (Bolin et al., 2009; Brantjes, 1980; Knoll, 1926; Kugler, 1970; Proctor et al., 1996; Vogel, 1961, 1965). The floral designs excel in diversity, and they are extremely variable in size, too. From small flowers of some <20 mm (e.g., *Ceropegia claviloba* Werderm.) to the giant inflorescences of titan arum (*Amorphophallus titanum* Becc.) that can reach up to 3 m, the largest angiosperm inflorescences (Davis et al., 2008).

Among the above mentioned plant families with flowers/blossoms to detain their pollinators, some taxa are mutualistic and offer rewards, e.g., oviposition sites, nutritious food-tissues, pollen, mating sites, and shelter (Diaz and Kite, 2006; Gottsberger, 1989; Sakai, 2002). In such taxa, the pollinators are not specifically hindered to exit the flower, instead, they "voluntarily" stay there. In contrast, non-rewarding deceptive species have special trapping devices making it impossible for the pollinator to leave, and the designs of trapping devices relate to the type of pollinator trapped (Bröderbauer et al., 2013). Such trap flowers show convergent features with high similarities in minute detail, though in some angiosperm lineages different plant parts are used to form non-homologous traps (Vogel, 1965).

By whatever strategy or mechanism pollinators are made to stay, they are not supposed to die inside the flowers (but see Vogel and Martens, 2000), but to exit them for pollen export to another flower, when, after a while, trapping devices become non-functional. Some deceptive trap flowers even provide small amounts of nectar, not regarded as true reward, to ensure pollinator survival (Dafni, 1984). The pollinators of deceptive trap flowers are exclusively insects, including beetles, bees, and flies (Gibernau, 2003; Renner, 2006). The overwhelming majority of trap flowers, e.g., *Aristolochia, Arisaema*, and *Ceropegia*, however, is fly pollinated (Renner, 2006).

The genus Ceropegia L.

Ceropegia L. (Apocynaceae, Asclepiadoideae) is one of the most species rich genera which evolved trap flowers (Coombs et al., 2011; Ollerton et al., 2009) that combine beauty and functionality with an extreme level of synorganisation (Masinde, 2004; Vogel, 1961). *Ceropegia* includes more than 200 described species restricted to Old World tropical and subtropical habitats. Highest diversification took place in South-East Africa, India, Madagascar and China (Meve and Liede-Schumann, 2007; Murthy et al., 2012). Within the Asclepiadoideae, myiophily is the basal and most common

pollination syndrome (Masinde, 2004; Ollerton and Liede, 1997), and the deceptive trap flowers of *Ceropegia* are pollinated by flies of various families (see below).

Ever since have the trap flowers of *Ceropegia* aroused the interest of naturalists, and first records on flies as pollinators date back more than a century (Delpino, 1869; Knuth, 1898-1905). However, pioneer studies on flower structure, functional flower parts, and pollination biology were contributed by Müller (1926) and above all by the late Stefan Vogel. Vogel (1954, 1960, 1961, 1965) provided very detailed and passionate descriptions on the various specialized flower parts and tissues and their functional interaction to achieve pollination by small flies.

Flower morphology

Despite the great diversity in size, coloration, shape, and ornamentation of *Ceropegia* flowers, the basic floral structure is similar in all species (see Vogel, 1961). The corona is fused to form a sophisticated pitfall flower with special radial symmetry, a so called “revolver flower” (Endress, 1994). The fused corolla is divided in three functional parts, all contributing to effective pollinator trapping: 1) the flower tip, often decorated with versatile hairs, provides entrance for flower visitors through five orifices, 2) the tube through which insects inadvertently drop down and are hindered to escape by downward-pointing hairs, and 3) the basal inflation (ostiolum) that contains the gynostegium (fused male and female reproductive organs), and where flower visitors are temporarily imprisoned (see Vogel, 1961). The maximum size of the flower visitor/pollinator is predefined by the flower size, i.e., the wideness of the distal orifices and the tube width.

Though several small arthropods can be found inside flowers (Ollerton, 1999; Ollerton et al., 2009; and pers. obs.), only dipterans have been described as effective pollinators of *Ceropegia* (Bayer, 1978; Coombs et al., 2011; Karuppusamy and Pullaiah, 2009; Ollerton and Forster, 1995; Ollerton et al., 2009; Sabrosky, 1987; Vogel, 1961). Whether a fly that entered the flower can act as pollinator depends on its strength and the morphology of its mouthparts (Masinde, 2004). A strong enough fly with appropriate membranes on its extended proboscis can pick up a pollinarium and/or deposit pollinia during the time it is trapped inside the inflation (Vogel, 1961). Thus, morphological traits from both the flower and the fly lead to pollinator specificity in *Ceropegia*. However, pollinator specificity cannot only be explained by morphological traits. Instead, other traits, such as floral scents, also seem to be

involved in attracting just a subset of potential pollinators (Heiduk et al., 2010; Vogel, 1961).

Flower scent

If perceivable to the human nose, the descriptions of floral scents in *Ceropegia* range from pleasantly sweet and fruity, via soapy and leather-like, to pungent acidic and disgusting putrid (Vogel, 1961; pers. obs.). Though some *Ceropegia* species smell alike, basically every species shows its own characteristic blend (Vogel, 1961). More than half a century ago, Vogel (1961) experimentally demonstrated that floral scent is produced and emitted by specialized epithelia (i.e., osmophores) at the flower tips, and this finding was later confirmed by Heiduk et al. (2010) with analytical techniques. *Ceropegia* species often grow hidden in bushes and thus, Vogel (1961) hypothesized that floral scent plays a major role in attracting the fly pollinators from a distance. He experimentally proved his hypothesis by offering flowers/flower parts in non-transparent vials to which flies were attracted.

It has been considered likely that *Ceropegia* flowers deceive pollinators through chemical mimicry. Possible models that have been suggested include sex pheromones, oviposition sites, and food sources (Vogel, 1961, 1993), and presumably dependent on the type of chemical mimicry specific pollinators are attracted.

Pollinating flies

The pollination biology of myiophilous *Ceropegia* flowers is a remarkable example of a highly specialized flower-fly relationship. In general, each *Ceropegia* species has a functional group of fly pollinators belonging to only one or few families and/or genera (Heiduk et al., 2010; Ollerton et al., 2009).

Diptera of various families, such as Ceratopogonidae, Chloropidae, Drosophilidae, Milichiidae, Phoridae, Scatopsidae, and Sciaridae pollinate *Ceropegia* flowers. The biology of the pollinating fly taxa is hyperdiverse, not only among but even within the different families. As adults, flies of many of these families are well known flower visitors and/or pollinators, pollen eaters, nectar feeders, blood suckers, scavengers, and detrivores on various organic materials (e.g., Larson et al., 2001, and references therein; Marshall, 2012). The reproductive and larval biology is even more diverse, and for many groups still undescribed. Several *Ceropegia* pollinating flies are saprophagous and as larvae depend on rotting organic matter, animals, or

animal secretions (Courtney et al., 2009; Sivinski et al., 1999, and references therein; Vogel, 1961, 1993). Adult flies have suctorial mouthparts and thus depend on liquid or liquifiable food sources (Corlett, 2004) but *Ceropegia* flowers are non-rewarding. They neither offer nectar nor small pollen grains that could be insalivated, which supports the assumption that *Ceropegia* flowers are deceptive and trick flies into pollination.

Among the pollinators of *Ceropegia* are also taxa with kleptoparasitic habits – they steal food from other predatory arthropods (e.g., spiders), by feeding on hemolymph or other secretions leaking from their prey items (Eisner et al., 1991; Robinson and Robinson, 1977; Sabrosky, 1983; Sivinski, 1985; Sivinski et al., 1999; Sivinski and Stowe, 1980). It is generally believed that kleptoparasitic flies find such prey items through volatile organic compounds (VOCs) generated either by the predator or released from wounds of prey items after a predator attack (Aldrich and Barros, 1995; Beavers et al., 1972; Eisner et al., 1991; Sivinski et al., 1999; Zhang and Aldrich, 2004). This information suggests that kleptoparasitic fly pollinators may mistake the flower scent for odor of a food source. This special kind of pollination system, called kleptomyiophily, was indeed recently discovered in an *Aristolochia* species with trap flowers (Oelschlägel et al., 2015). Regarding *Ceropegia*, nothing is known about their pollination strategies and the models mimicked by the flowers through their floral scent. Only for *C. dolichophylla* do data suggest that kleptomyiophily might also occur in *Ceropegia*. However, clear proof was lacking and the pollination strategies in *Ceropegia* remain puzzling; an exciting field ripe for exploitation.

Aims of my research

In my PhD project, I tried to find answers to many of the questions unsolved since Vogel's hypotheses made in 1961. In detail, I investigated 14 *Ceropegia* species and the specific questions of my research to elucidate their pollination strategies were:

1) What are the visiting and pollinating flies?
2) What is the reproductive success of the species?
3) Which compounds are released by the flowers?
4) Are there correlations between floral chemistry and pollinating flies?
5) Is there a phylogenetic signal in floral scent and in visitor/pollinator assemblages?
6) What are the mimicry strategies of studied species?

To answer these questions, I collected flower visitors of and determined pollinator assemblages in the 14 studied species. To gain information about the reproductive success of *Ceropegia*, I calculated “pollen transfer efficiency” (PTE) in eight of the 14 species. For all species, I collected floral scent with headspace techniques, and analyzed the scent compositions by gas chromatography coupled to mass spectrometry (GC/MS). For *Ceropegia dolichophylla* and *C. sandersonii* I identified the components which can be perceived by their fly pollinators using gas chromatography-electroantennographic detection (GC/EAD), and further assessed the behavioral activity of selected electrophysiologically active components (singly and in mixtures) in field bioassays. Moreover, I calculated the genetic distances among all studied species and used them to check for phylogenetic signals in floral scent chemistry and visitor/pollinator assemblages. Further on, I correlated the multivariate data sets of floral scent components with pollinating fly morphospecies.

Materials and Methods

Plant material and study sites ***(Publications 1, 2, 3)***

A total of 14 *Ceropegia* species (Figure 1) were studied regarding their pollination biology and chemical ecology of pollination. Most species were investigated in their natural habitats. For some species only green house plants within and/or far away from natural habitats were available, and for a subset of species both cultivated and field plants were studied. For all studied species, information on natural distribution, study sites, and investigated aspects are gathered in Table 1.

Figure 1: Flowers of investigated *Ceropegia* species. **A:** *Ceropegia rupicola*; **B:** *C. stenantha*; **C:** *C. ampliata*; **D:** *C. denticulata*; **E:** *C. barklyi*; **F:** *C. carnosa*; **G:** *C. haygarthii*; **H:** *C. woodii*; **I:** *C. crassifolia*; **J:** *C. nilotica*; **K:** *C. pachystelma*; **L:** *C. cycniflora*; **M:** *C. dolichophylla*; **N:** *C. sandersonii*. Photographs: U. Meve, A. Heiduk, and S. Dötterl. **(Based on Figure 1 in Publication 3)**

Table 1: Natural distribution (according to Meve (2002) and recently retrieved data), study sites, and investigated aspects of studied *Ceropegia* species. AT: Austria; BF: Burkina Faso; DE: Germany; RSA: Republic of South Africa; EAD: Electroantennographic detection; PTE: Pollen transfer efficiency; ○: non-natural habitat; ●: natural habitat (in RSA: KwaZulu-Natal; in China: Fanjing Mountain).

***Ceropegia* species**	**Natural distribution**	**Study site**		**Floral scent**	**Pollinators**	**PTE**	**EAD-/behaviorally active compounds**
		●	○				
C. ampliata E. Mey. subsp. *ampliata*	Botswana, Kenya, Mozambique, RSA	RSA	DE	○	○		
C. barklyi Hook. f.	RSA	RSA		●	●	●	
C. carnosa E. Mey.	Kenya, RSA, Swaziland, BF	RSA, BF		●	●	●	
C. crassifolia Schltr.	Namibia, Kenya, Botswana, Zimbabwe, RSA, Swaziland	RSA		●	●	●	
C. cycniflora R.A. Dyer	RSA	RSA		●	●		
C. denticulata K. Schum. ex Engl.	Kenya, Tanzania, Uganda, RSA		DE	○			
C. dolichophylla Schltr.	China (Yunnan)	China	DE	●,○	●,○	●	○ / ●,○
C. haygarthii Schltr.	Angola, Mozambique, RSA	RSA		●	●	●	
C. nilotica Kotschy	Tropical & subtropical Africa (from Senegal to RSA)	RSA		●	●	●	
C. pachystelma Schltr.	Mozambique, Namibia, Botswana, Zimbabwe, RSA, Swaziland	RSA		●	●	●	
C. rupicola Defl.	Yemen		AT	○	○		
C. sandersonii Decne. ex Hook.	RSA, Mozambique, Swaziland	RSA	AT, DE	●,○	●,○		○ / ●,○
C. stenantha K. Schum.	South & East Africa	RSA	DE	○	●,○		
C. woodii Schltr.	RSA, Swaziland, Zimbabwe	RSA		●	●	●	

Determination of pollinators ***(Publications 1, 2, 3)***

To gather information on flower visitors and pollinators of studied *Ceropegia* species, flowers were collected during the day and either frozen or stored in ethanol for later investigation. In the lab, flowers were opened carefully, and insects inside the flowers were removed and examined for pollinaria (corpusculi with no, one, or two pollinia) clipped to their bodies. Flower visitors that carried pollinaria were denoted as pollinators (see Ollerton et al., 2009). All insects were stored in a 4% solution of glycerin in ethanol (99.8%), and dipterans were later identified to family, genus, and/or (morpho)species level by qualified fly taxonomists.

Pollen transfer efficiency (PTE) ***(Publications 1, 3)***

Pollen transfer efficiency was determined for *C. dolichophylla*, *C. barklyi*, *C. crassifolia*, *C. haygarthii*, *C. nilotica*, *C. pachystelma*, *C. carnosa*, and *C. woodii* in their natural habitat. For that purpose flowers were picked from plants in the afternoon/evenings, and gynostegia were checked for pollinaria removal and pollinia insertion. For each species the mean number of inserted pollinia and the mean number of removed pollinaria were calculated. Reproductive success was then determined as the percentage of removed pollinia that were inserted between guide rails (see Coombs et al., 2009; Johnson et al., 2005). Since in *Ceropegia* each pollinarium consists of two pollinia, the mean number of inserted pollinia was divided by twice the mean number of removed pollinaria. For calculation of pollen transfer efficiency (PTE) the following formula was applied:

$PTE = p_i/(2xP_r)$,

thereby, p_i is the mean number of inserted pollinia, and P_r is the mean number of removed pollinaria (see Johnson et al., 2005; Johnson et al., 2004; for application in *Ceropegia* see Coombs et al., 2011).

Dynamic headspace ***(Publications 1, 2, 3)***

Volatiles emitted by *Ceropegia* flowers were collected using standard approaches (Dötterl et al., 2005) with two different dynamic headspace methods. Thermal desorption (TD) samples were collected for identification of scent compounds, while solvent acetone (SAc) samples were collected for electrophysiological analyses.

For TD-samples, individual, newly opened flowers (one per sample or in some cases a group of flowers) were enclosed in situ in polyester oven bags (Toppits®, Germany) for 5 min to 110 min (see Heiduk et al., 2010), depending on the intensity of scent as perceived by the human nose. The accumulated floral volatiles were then trapped by pulling air from the bag through small adsorbent tubes for 2 min to 30 min (see Heiduk et al., 2010) using a membrane pump (G12/01 EB, Rietschle Thomas Inc., Puchheim, Germany). The flow rate was adjusted to 200 ml/min by using a flow meter. The adsorbent tubes were made of ChromatoProbe quartz microvials of Varian Inc. (length: 15 mm, inner diameter: 2 mm), from which the closed end was cut off. These tubes were filled with a mixture of 1.5 mg Tenax-TA (mesh 60-80) and 1.5 mg Carbotrap B (mesh 20-40, both Supelco), which was fixed by using glass wool. To distinguish between floral and ambient air components, the surrounding air was collected simultaneously.

For SAc-samples, the flowers were enclosed and floral volatiles were pulled from the bag as described for TD-samples. However, volatiles were not accumulated but constantly trapped for several hours (see Heiduk et al., 2010) into larger adsorbent tubes (glass capillaries; length: 8 cm, inner diameter: 2.5 mm) containing 15 mg Tenax-TA (mesh 60–80) and 15 mg Carbotrap B (mesh 20–40) at a flow rate of 100 ml/min. The trapped volatiles were then eluted from each adsorbent tube with 60-70 µl of acetone (SupraSolv, Merck KgaA, Germany; following Dötterl et al., 2005).

A setup different from that for floral scent sampling was used for in situ sampling of volatiles emitted from honeybees (*Apis mellifera carnica* Poll./*A. m. ligustica* Spin., and *A. m. scutellata* Lep.) under simulated attack in the lab (see Publication 2, and Figure 2). Other than described for sampling flowers, the individual bees were not put into polyester oven bags but inserted, abdomen first, into glass tubes (inner diameter: 5.4 mm). To simulate predator attack, the bees were “attacked” with the tip of a glass pipette, and at that provocation the bees a) bit into the glass pipette, and b) extruded their stings and tried to pierce the glass tube. TD-samples of the volatile components released from these bees were collected for two minutes per bee in an adsorbent trap which was coupled to the glass tube at the side of the bee’s abdomen. To specify volatile emission induced by the simulated attack, control samples were taken from each bee individual before “attacking” it with a glass pipette (*A. m. carnica/ligustica*). For samples of

A. m. scutellata an ambient control sample was used to specify volatiles emitted due to the simulated attack. SAc-samples of honeybees were collected with a similar setup. However, only the bee's abdomen was inserted into a glass tube and predator attack was simulated by gently squeezing the bees between forefinger and thumb. The emitted volatiles were collected into a larger adsorbent tube (the same as described above), and eluted from each tube with 70 µl of acetone.

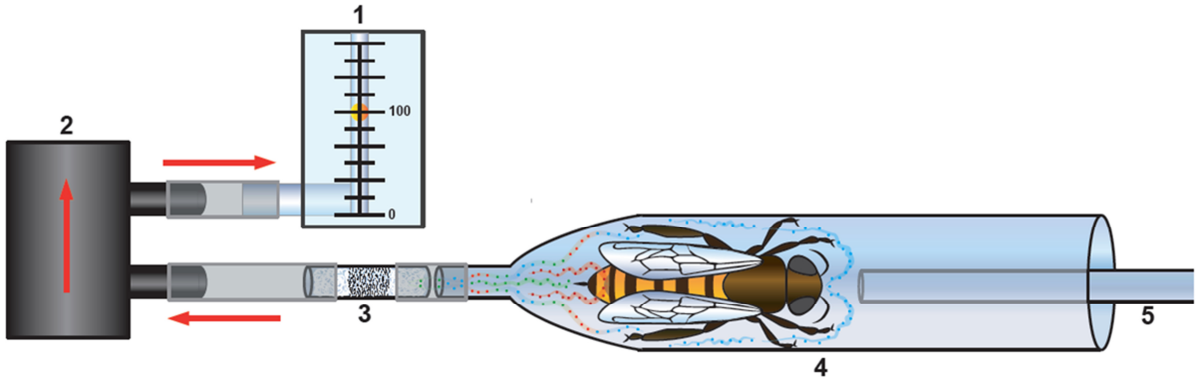


Figure 2: Experimental setup for collection of volatiles from honeybees under simulated attack. **1:** Flow meter; **2:** pump; **3:** adsorbent tube; **4:** glass tube with honey bee; **5:** glass pipette to simulate an attack. Flow meter, pump, adsorbent tube, and glass tube with honeybee are connected via silicone tubes. Red arrows indicate direction of air flow. Colored dots indicate volatile molecules released from sting (green), Nasonov (red), and mandible (blue) glands. **(Supplemental Figure S2 in Publication 2)**

Gas chromatography/mass spectrometry (GC/MS) ***(Publications 1, 2, 3)***

Volatile samples (TD- and SAc-samples) were analyzed by gas chromatography (GC) coupled to mass spectrometry (MS). While volatile components can be separated via GC with great resolution, MS allows identifying single components by providing detailed structural information as basis for identification (Hites, 1997).

Samples were collected at multiple different locations in Germany, Austria, South Africa, and China, and analyzed over several years in different labs with different equipment (see also Heiduk et al., 2010). As shown in previous studies, analytical equipment has little influence on the results of scent blend analyses (Johnson, pers. comm.). Details on the different GC/MS setups and settings are given in Publication 3. The resulting GC/MS data were processed with the appropriate software package of each GC/MS setup.

Identification of volatile components from GC/MS spectra based on NIST 11, Wiley 9, MassFinder 3, FFNSC 2, and Adams data bases (Adams, 2007). Whenever possible, compounds were verified by comparison with published Kovats retention indices (KRI) or by retention times and mass spectra of authentic standards (available in the plant ecology lab of the University of Salzburg and South Africa). Total scent emission was estimated by injecting known amounts of monoterpenoids, aromatics, and aliphatics (applied to small adsorbent tubes) into the GC/MS, and quantification then based on the mean peak area of the injected components.

Electrophysiological analyses (GC/EAD) ***(Publications 1, 2)***

GC/EAD was applied to detect floral components which can be perceived by pollinating flies. The floral scents (SAc-samples) of *C. dolichophylla* and *C. sandersonii* were tested on antennae of *Desmometopa* flies. Furthermore, the volatile sample of honeybees under simulated attack (see above) was tested on pollinators of *C. sandersonii*.

For measurements, the head of a fly was cut off and placed between two glass micropipette electrodes to establish an electrical potential. Whenever the antenna perceives a scent component, i.e., has receptors for a specific molecule, the EAD detects a current flux which gets visible as a peak in the resulting electroantennogram. For identification of the EAD-active components, the tested SAc-samples were analyzed by GC/MS (see above). The acquired knowledge about electrophysiologically active components in floral scent and honeybee samples facilitated the experimental identification of behaviorally active (i.e., attractive) components.

Behavioral studies ***(Publications 1, 2)***

Floral scent components of *Ceropegia sandersonii* and *C. dolichophylla,* which were EAD-active in pollinating *Desmometopa* flies, were tested for their attractiveness in the field. Therefore, synthetic standards of selected components were used as acetone solutions. The synthetic standards were either commercially available (Sigma-Aldrich, Merck) or synthesized by chemists (for details see Publications 1, 2). Single components and mixtures thereof were offered in glass vials tucked into the ground or mounted to sticky traps (see Publication 2). The performed assays varied from two choice to several choice assays, and in each assay a glass vial containing

mere acetone was used as control sample. Flies attracted to a sample were caught with an insect net when whirring above the vials or when they landed within a distance of ~10 cm from the vial. Flies attached to sticky traps were carefully removed and rinsed with petrol to remove the insect glue. All flies were stored in a 4% solution of glycerin in ethanol (99.8%) for later identification by fly taxonomists.

Genetic relatedness of study plants ***(Publication 3)***

To allow conclusions about the genetic relatedness of studied *Ceropegia* species, leaf material was collected, mostly from accessions for which scent was studied. For detailed processing of DNA sequences and resulting data, see Publication 3. To visualize the genetic relatedness, a Maximum Likelihood (ML) tree was calculated on the CIPRES Platform (Miller et al., 2010) using RAxML-HPC v. 8.2.9 (Stamatakis, 2014) on BlackBox with a mixed partition model for the six data partitions (Internal Transcribed Spacer (ITS) of nrDNA, five chloroplast DNA markers: *trn*T-L, *trn*L-F and *trn*H-psbA spacers, *trn*L and *rps*16 intron), and standard settings.

Statistical analyses

Floral scent **(Publications 1, 2, 3)**

To determine (semi-)quantitative differences in floral scent profiles among the 14 studied *Ceropegia* species, the Bray-Curtis (BC) similarity index calculated in Primer 6.1.11 (Clarke and Gorley, 2006) was used.

Since emission rates of flowers were variable both among and within species, the (semi-)quantitative differences were based on the relative contribution (percentage of the total peak area) of each component to the total amount of scent. Where required, mean relative amounts per species were calculated. In cases where more than one flower per plant individual was sampled, the mean relative amount per individual was calculated. Whenever multiple samples were taken from a single flower, mean relative amounts per flower were built.

To test for differences in scent among species, an ANOSIM (Factor: Species; 10000 permutations) was performed based on the BC-matrix, using again Primer 6.1.11. Nonmetric multidimensional scaling (NMDS) was used to visualize similarities/dissimilarities among individual samples and different species (Clarke and Gorley, 2006). In the NMDS, a stress value is given to assess how well the distance matrix is resembled by the particular ordinance of samples. The smaller the stress

value, the better the accordance of the ordinance to the reproduced distance matrix (Clarke, 1993). *C. dolichophylla* was sampled in China at two different sites (see Publication 1) and to test for an Area effect in floral scent, a PERMANOVA (Factor: Area; 10000 permutations) was performed in Primer, including the add-on package Permanova+ 1.0.1 (Anderson et al., 2008; Clarke and Gorley, 2006). Additionally, quantitative differences in absolute amounts of scent between the two sampling sites were tested applying a t-test (StatSoft Inc., 2008). Beforehand, normality was tested using Shapiro-Wilk test, and homogeneity of variances was tested by Hartley's test (StatSoft Inc., 2008).

Relationships between genetic relatedness, floral scent, and flower visiting/pollinating flies **(Publication 3)**

It was tested whether phylogenetic relatedness of studied *Ceropegia* species explained floral scents and flower visiting/pollinating flies. To estimate such phylogenetic signal in the high-dimensional, multivariate dataset of floral scents, and in the multivariate datasets of flower visiting/pollinating fly taxa (family and morphospecies level) K_{mult} (Blomberg et al., 2003) was used, a generalized variant of multivariate Blomberg's K (Adams, 2014; Blomberg et al., 2003, K_{mult}). A K_{mult} value of 1 indicates the expected trait evolution under Brownian motion. By running 999 permutations the significance of phylogenetic signal was determined relative to the expected evolution.

Linkage between floral scent and fly taxa was tested by correlating a BC similarity matrix based on floral scent (species means in relative amounts per compound were used for calculation) to BC similarity matrices of fly visitors and fly pollinators (family and morphospecies level) using RELATE in Primer (Spearman's rank correlation, 10000 permutations).

Whenever fly visitors/pollinators from both the native and the non-native range were available, only data from the native range were used for calculations.

Bioassays **(Publication 2)**

Generalized linear models implemented in SPSS 21 (IBM Corp.) were applied to analyze bioassays performed with synthetic floral components of *C. sandersonii*.

Results and Discussion

Pollen transfer efficiency, flower visitors and pollinators ***(Publications 1, 3)***

In studied *Ceropegia* species, pollen transfer efficiency was generally low (0%-7%) and in accordance with data published for *C. ampliata* (Coombs et al., 2011). For *C. pachystelma* however, PTE was considerably high and yielded 33%, probably due to a much higher number of simultaneously open flowers when compared to the other species. Regarding other plants, a reasonable comparison would be with other Apocynaceae or the Orchidaceae, as they are the only ones having their pollen packed to pollinia. Within Apocynaceae, PTE seems to be variable with values from lower than 10% (Liede, 1994; Liede and Whitehead, 1991; Ollerton et al., 2003) to nearly 40% (Coombs and Peter, 2010; Forster, 1994). Orchids pollinated by insects generally show values lower than 10% when they are deceptive (e.g., Craig and Johnson, 2009; Johnson et al., 2005; Johnson et al., 2004), whereas pollen transfer efficiency in rewarding orchids is nearly four-fold higher (Craig and Johnson, 2009). In the bird pollinated and rewarding orchid *Disa chrysostachya* Sw. PTE, however, was also lower than 10% (Johnson and Brown, 2004) which points out that not only the presence and absence of reward but also the type and behavior of the pollinator impacts the efficiency of pollen transfer. However, PTE is primarily influenced by how pollen is presented to the pollinator. This becomes most obvious when considering that PTE of plants with granular pollen is generally many times lower than that of Orchidaceae or Apocynaceae (Harder, 2000; Harder and Johnson, 2008). This suggests that the packing of pollen into discrete pollinia positively influences pollen fate by reducing pollen loss and enhancing export with successful deposition, even independent of the type of pollinator used (Harder and Johnson, 2008).

The flowers of investigated *Ceropegia* species were exclusively visited/pollinated by taxa of ten different fly families, namely Anthomyiidae, Ceratopogonidae, Chloropidae, Drosophilidae, Lauxaniidae, Milichiidae, Muscidae, Phoridae, Scatopsidae, and Tachinidae. Based on flowers with positive trap catches, a single flower contained a mean number of four flies. The maximum number of fly individuals collected from a single flower was 54 in *C. nilotica*.

In total, at least 40 different dipteran morphospecies were collected from the flowers, and all flies were generally small in size (<3 mm) as already described in previous studies on *Ceropegia* pollination (Heiduk et al., 2010; Karuppusamy and Pullaiah, 2009; Masinde, 2004; Ollerton et al., 2009; Vogel, 1961). Only in

C. ampliata the visitors/pollinators were bigger than 3 mm which also is in consistence with literature data (Coombs et al., 2011). At morphospecies level, flower visitors generally differed among the *Ceropegia* species. However, there was an overlap at genus and family level among some species, and there was a phylogenetic signal in flower visitors on family level (K_{mult}=0.736, p=0.003).

Pollinators could be identified for all studied species, except for *C. denticulata*. A total of 33 fly morphospecies spread across eight of the ten above mentioned families (not Lauxaniidae and Muscidae) had pollinaria attached to their mouthparts, and were thus determined as legitimate pollinators. Pollinaria were never found to be attached on any other body part than mouthparts of the flies, as also described in other studies on *Ceropegia* pollinators (e.g., Coombs et al., 2011; Karuppusamy and Pullaiah, 2009; Masinde, 2004). In general, the flies had only a single pollinarium attached, but two Drosophilidae collected from flowers of *C. crassifolia* carried two and four pollinaria, each; and one drosophilid fly collected from a flower of *C. rupicola* carried three pollinaria, i.e., six pollinia (Figure 3).

Several of the pollinating fly families are also described as pollinators of other asclepiads (Meve and Liede, 1994; Nihei and Schwarz, 2011; Ollerton et al., 2003; Ollerton and Liede, 1997; Ollerton and Liede, 2016; Yassin et al., 2012) and/or other plant families (e.g., Corlett, 2004; Larson et al., 2001; Orford et al., 2015). However, regarding *Ceropegia*, most of the 33 pollinating fly morphospecies were not listed as pollinators before (Ollerton et al., 2009), and ten out of 18 pollinating genera are described for the first time as pollinators in *Ceropegia*. On family level, however, the flies (not Anthomyiidae) are already known to pollinate the same (*C. ampliata*, *C. carnosa*, *C. crassifolia*, *C. denticulata*, *C. haygarthii*, *C. nilotica*, *C. stenantha*, and *C. woodii*) or other *Ceropegia* species within and outside their native ranges (Bhatnagar, 1986; Coombs et al., 2011; Ollerton and Forster, 1995; Ollerton et al., 2009; Vogel, 1961).

For nine studied species the pollinators belonged to only a single fly family (e.g., Scatopsidae in *C. stenantha*, Milichiidae in *C. cycniflora*), and four species were pollinated by taxa of two families (Figure 4). This finding shows a high functional specialization on fly pollinators, and such affinities have also been determined in other species of *Ceropegia* (Ollerton et al., 2009). The functional pollinator group, however, often comprised several species, and the ecological specialization therefore seems to be less distinctive. The widest range of flower

visitors was found in *C. stenantha*; flowers of this species contained 12 fly species (only native range). Except for three flies, all specimens were Scatopsidae of four genera, and individuals of four species in three of these genera carried pollinaria.

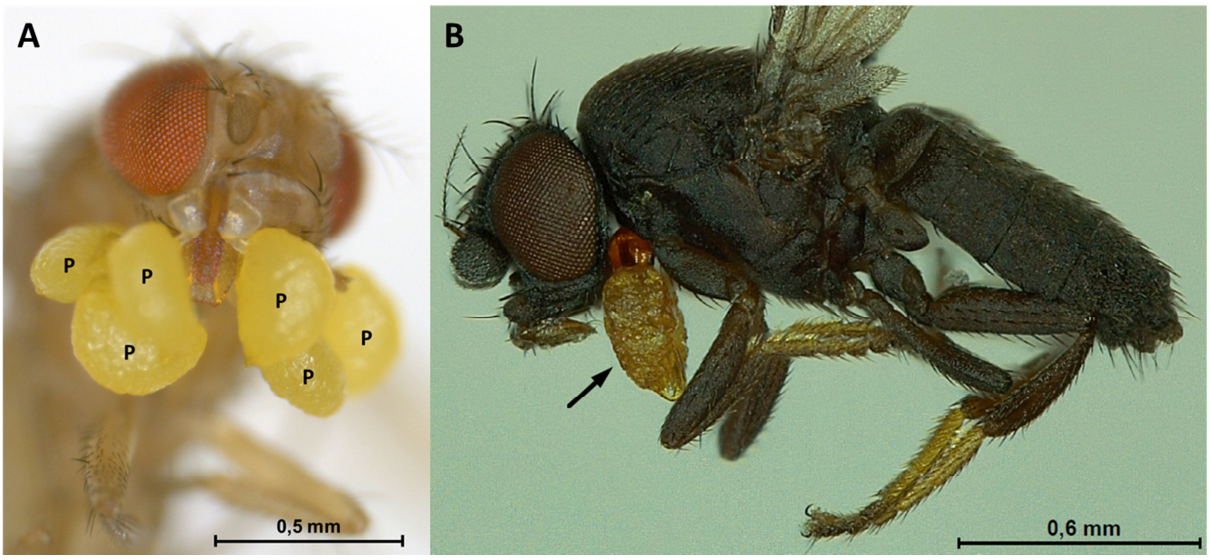


Figure 3: Pollinators of *Ceropegia* with pollinaria attached to their mouthparts. **A:** Close up of the head of *Drosophila melanogaster* collected from a flower of *C. rupicola*, Salzburg, Austria. P: Pollinium. **B:** *Desmometopa* cf. *nudigena* collected from a flower of *C. sandersonii* in KwaZulu-Natal, South Africa. Arrow indicates the pollinarium. **(B: Supplemental Figure S1A in Publication 3)**

The pollinator assemblages used by the *Ceropegia* species showed phylogenetic signal both on family level (K_{mult}=0.725, p=0.019) and on morphospecies level (K_{mult}=0.749, p=0.003). This relation is most obvious in the closely related species *C. barklyi* and *C. woodii*, which are pollinated by Ceratopogonidae (Figure 4). Thus, the pollinator assemblage seems to be an evolutionary inflexible trait. Flowers of *C. barklyi* and *C. woodii* are among the most filigree *Ceropegia* flowers with markedly narrow tubes. Their micro-dipteran pollinators of the family Ceratopogonidae were found to visit other *Ceropegia* species, e.g., *C. haygarthii*, *C. nilotica*, and *C. pachystelma*, as well. However, these flies only carried pollinaria when collected from the small flowering species *C. barklyi, C. pachystelma*, and *C. woodii*. Though, such small flies have access to flowers with relatively broad tubes, their mouthparts and strength probably only match up to gynostegia of small sized *Ceropegia* species. It is worth mentioning here, that the size of pollinating flies matches the size and tube width of flowers. Small flowers with extremely narrow tubes are pollinated by tiny Diptera, whereas flowers with extraordinary broad tubes (*C. ampliata*) are pollinated

by big sized calyptrate fly taxa. As also discussed for *Aristolochia* (Brantjes, 1980; Rulik et al., 2008), tube width of *Ceropegia* flowers might act as a filter for apt sized pollinators, and could give a hint on possible pollinating fly taxa at least in some cases. Congruously, it can be assumed that the phylogenetic signal in pollinator assemblages is to a certain degree a result of evolutionary influenced flower morphology. However, key role in attraction of the appropriate pollinator is still considered to be played by floral scent. This is because *Ceropegia* flowers do not morphologically but chemically resemble their mimicked model (see below), and floral scent alone, decoupled from any visual cues, selectively attracts the specific pollinating flies (Heiduk et al., 2010; Vogel, 1961).

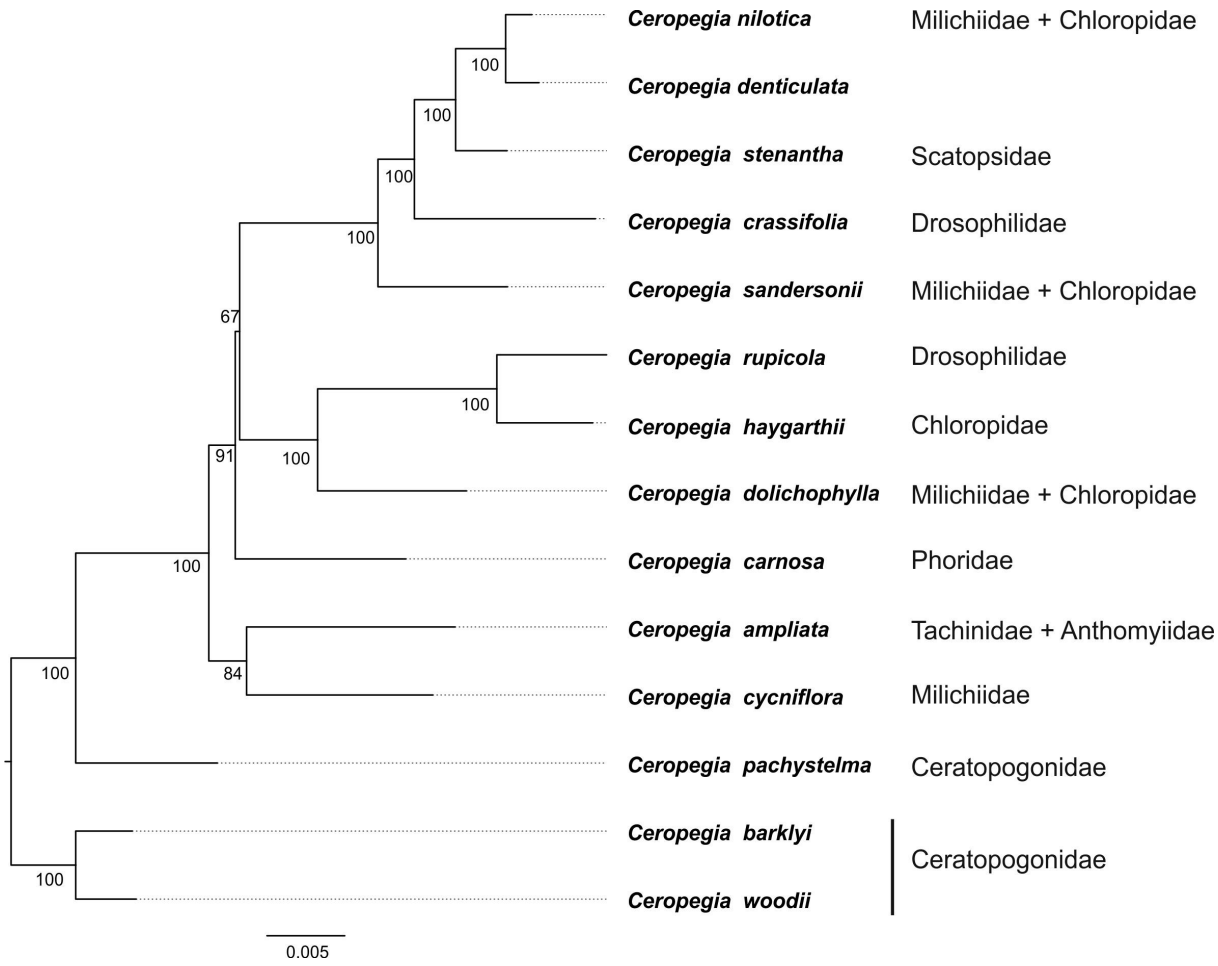


Figure 4: Phylogenetic tree based on Maximum Likelihood analysis of six markers of nuclear and cpDNA. Numbers below branches indicate frequency of bipartitions. Pollinating fly families are indicated for each species except for *C. denticulata*, for which no data on fly pollinators are available. **(Figure 2 in Publication 3)**

Floral scent ***(Publications 1, 2, 3)***

Floral scents of the 14 investigated *Ceropegia* species comprised 317 different components of which 169 were identified. These components belong to several compound classes, mainly to aliphatic and aromatic compounds, and terpenoids. The floral bouquets comprised widespread floral volatiles, such as linalool, (*E*)-ß-ocimene, benzaldehyde, and 2-heptanone that have already been reported in the floral scent of many flowering plants (Knudsen et al., 2006). Other components, such as 2-nonyl acetate (listed as 2-acetoxynonane in Publication 2), (*E*)-2-octenyl acetate, (2*S*,6*R*,8*S*)-8-methyl-2-propyl-1,7-dioxaspiro[5.5]undecane, and N-(3-methylbutyl)acetamide were not identified in any plants other than *Ceropegia* before (see Knudsen et al., 2006). Furthermore, the aromatic components 3-acetyloxy-4-phenylbutan-2-one and 3-acetyloxy-1-phenylbutan-2-one, identified in *C. stenantha* (Publication 3), have not even been reported from nature before. These components were already synthesized by collaborating chemists; however, their attractiveness to scatopsid fly pollinators of *C. stenantha* is still to be tested.

The floral scent bouquets of studied *Ceropegia* species showed remarkable interspecific differences (Figure 5A, B). There was high variability in the total amount of scent (minimum of 1 ng/15 min in *C. pachystelma*; maximum of >1000 ng/15 min in *C. cycniflora*), the total number of compounds (minimum of five in *C. woodii*, *C. crassifolia*, and *C. pachystelma*; maximum of 56 in *C. sandersonii*), and the overall scent chemistry.

The observed differences in floral scent among species were independent of genetic relatedness of the study plants, and there was no phylogenetic signal in floral scents (K_{mult}=0.316, p=0.628). Most obviously did the closely related species *C. crassifolia*, *C. denticulata*, and *C. stenantha* (Figure 4) differ in their scent patterns with clear differences in main floral volatiles (*C. crassifolia*: acetoin; *C. nilotica*: ethyl dodecanoate; *C. stenantha*: benzaldehyde). On the other hand, the less closely related species *C. denticulata* and *C. sandersonii* converged in their scents in that they both emit 2-heptanone, 2-heptanol, and 2-nonanol as dominant scent components. These results reveal that, in contrast to pollinator assemblages, this trait is evolutionary flexible.

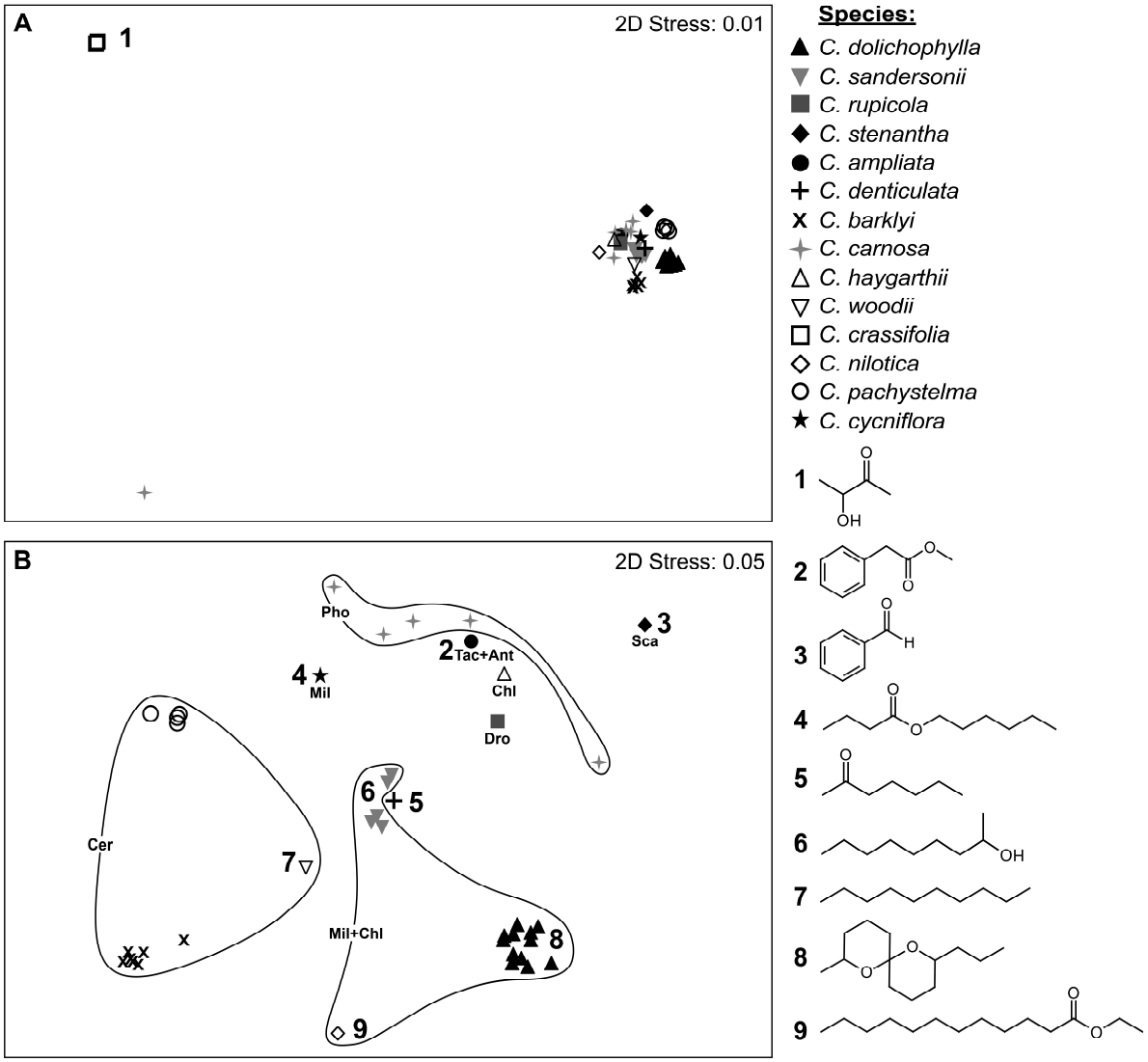


Figure 5: Non-metric multidimensional scaling (NMDS) of scent samples collected from studied *Ceropegia* species based on semi-quantitative Bray-Curtis similarities. **A:** NMDS including all samples from the investigated species. **B:** NMDS excluding *C. crassifolia* and two outlier samples of *C. carnosa*. Chemical structures of identified main scent components are given as **1:** acetoin; **2:** methyl phenylacetate; **3:** benzaldehyde; **4:** hexyl butyrate; **5:** 2-heptanone; **6:** 2-nonanol; **7:** decane; **8:** (2*S*,6*R*,8*S*)-8-methyl-2-propyl-1,7-dioxaspiro[5.5]undecane; **9:** ethyl dodecanoate. Pollinating fly taxa are indicated as **Ant:** Anthomyiidae; **Cer:** Ceratopogonidae; **Chl:** Chloropidae; **Dro:** Drosophilidae; **Mil:** Milichiidae; **Pho:** Phoridae; **Sca:** Scatopsidae; **Tac:** Tachinidae. **(Figure 3 in Publication 3)**

Studies on how phylogeny influences floral chemistry are yet rare and discordant. Whilst in some studies floral scent patterns correlate with phylogeny (e.g., Feulner et al., 2014), other studies showed that phylogenetic signal in scent chemistry depends on the time of scent emission (signal in scents released at night; no signal in scents released during daytime in Sileneae species) and pollinator guilds (Prieto-Benítez et

al., 2016). Overall, floral scent seems to be modulated both by phylogenetic relatedness and by pollinator-driven ecological selection (see Ho et al., 2016).

Compared to the interspecific variability in floral scent, the intraspecific variability was generally low (Figure 5A, B) in those *Ceropegia* species for which more individuals were tested. However, plants of *C. carnosa* did show a high intraspecific variability (Figure 5B) along with low absolute amounts of scent. Flowers of *Ceropegia* usually emit floral scent during daytime with species specific timeframes of peak emission rates (Vogel, 1961; and pers. obs.). In *C. carnosa* this timeframe was possibly missed when flowers were sampled, explaining the weakness of samples combined with disparity in scent composition.

Pollinator specificity through floral scent chemistry – mimicry strategies ***(Publications 1, 2, 3)***

My results show that pollinator assemblages in *Ceropegia* are highly specific and influenced by phylogenetic relatedness, whereas floral scent did not show a phylogenetic signal. At large, scent components and pollinator taxa were species specific, and overlap between floral scent and pollinating taxa was limited. Only *C. denticulata* and *C. sandersonii* overtly converged in their scent composition and shared flower visitors/pollinators. There was no correlation between floral scent and pollinator assemblages; nevertheless it can be assumed that pollinator specificity results from distinct scent chemistry. As mentioned above, *Ceropegia* uses a great variety of pollinating fly taxa with diverse biology. Considering the fact that, despite a phylogenetic signal in pollinator assemblages, closely related species attract distinct pollinators, it can be assumed that floral scent is under divergent selection pressure from pollinators. The specialized feeding habit and/or reproductive biology of pollinating fly taxa could have been the incitement leading to mimicry strategies where distinct floral scents are geared to attract specific fly taxa. Some species, such as *C. sandersonii* and *C. dolichophylla*, attract the same functional group of pollinators (*Desmometopa* spp.) with highly distinct scent components. Thus, despite their different scent chemistry, their floral odors address the same fly group.

Electrophysiological as well as behavioral studies showed that *C. dolichophylla* and *C. sandersonii* address their *Desmometopa* pollinators by species specific floral key components (see Publications 1 and 2) with overall only two out of 106 compounds overlapping between the species (see Publication 3). The plants differently exploit

the olfactory preferences of *Desmometopa* flies by emitting volatiles linked to different food sources of the flies. In *C. sandersonii* analyses of floral scent revealed that flowers emit volatiles also released by distressed honeybees (see publication 2), a common food source of *Desmometopa* flies (Biró, 1899; Landau and Gaylor, 1987; Mik, 1898). The overlap of compounds between flowers and distressed bees was 60%, including all main floral compounds such 2-heptanone, 2-heptanol, 2-nonanol, and (*E*)-2-octenyl acetate. Electrophysiological and behavioral analyses showed that the kleptoparasitic pollinators use such volatiles to locate bees under attack as a food source. *C. sandersonii* taps into this communication channel and tricks the flies into pollination by emitting a scent profile highly similar to that of a bee under attack. This special type of food source mimicry is called kleptomyiophily, a strategy recently also described for *Aristolochia rotunda* L. (Aristolochiaceae). The deceitful trap flowers of this plant mimic volatiles (e.g., hexyl butyrate, (*E*)-2-hexenyl butyrate, (*E*)-2-hexenyl hexanoate) of distressed Miridae bugs and are pollinated by kleptoparasitic Chloropidae (Oelschlägel et al., 2015).

Flowers of *C. dolichophylla* emit unusual floral volatiles (see Publication 1 and 3), two of which (i.e., 8-methyl-2-propyl-1,7-dioxaspiro[5.5]undecane and N-(3-methylbutyl)acetamide) were not known as plant volatiles before. From the 52 floral components of *C. dolichophylla*, 22, among them the unusual ones, can be perceived by its kleptoparasitic *Desmometopa* pollinators, and the two components (2*S*,6*R*,8*S*)-8-methyl-2-propyl-1,7-dioxaspiro[5.5]undecane and N-(3-methylbutyl)acetamide, either alone or in combination, turned out to be highly attractive to the flies (see Publication 1).

From the EAD-active scent components, 8-methyl-2-propyl-1,7-dioxaspiro[5.5]undecane, (*E*,*E*)-2,8-dimethyl-1,7-dioxaspiro[5.5]undecane, 2-ethyl-7-methyl-1,6-dioxaspiro[4.5]decane, and N-(3-methylbutyl)acetamide also occur in the venom of paper wasps (see Bruschini et al., 2006) and/or in cephalic secretions of *Andrena* bees (Bergström et al., 1982; Francke et al., 1981). These components might be key signals used by kleptoparasitic flies for food source location. Thus, *C. dolichophylla* most certainly also exploits the food seeking behavior of its kleptoparasitic *Desmometopa* pollinators.

So far, *C. dolichophylla* and *C. sandersonii* are the only species for which specific floral volatiles were assigned to pollinator attraction, and for which the mimicry strategy and the (potential) model mimicked could be uncovered. For other

Ceropegia species the floral components responsible for pollinator attraction and the pollination strategies yet remain unknown. Nevertheless, data on scent profiles and pollinating flies of studied species allow speculations about the deceptive strategies (see Publication 3).

For other studied *Ceropegia* species visited/pollinated by kleptoparasitic milichiid and chloropid flies, such as *C. cycniflora*, *C. denticulata*, *C. haygarthii*, and *C. nilotica*, a kleptomyiophilous pollination strategy is also reasonable (see Publication 3). As mentioned earlier, *C. denticulata* and *C. sandersonii* show similarity in floral scent. From the components emitted by *C. denticulata*, 18 (33%) are also emitted by *C. sandersonii*, however, in different quantities. Furthermore, flowers of *C. denticulata* are also visited, and most likely pollinated, by *Desmometopa* flies. It therefore seems plausible that *C. denticulata* not only also is kleptomyiophilous, but even mimics the same model as *C. sandersonii*, i.e., distress honeybees.

Floral scent of *C. cycniflora* almost entirely consists of compounds (e.g., hexyl butyrate, (*E*)-2-hexenyl acetate, (*E*)-2-hexenyl butyrate, and (*E*)-2-octenyl butyrate) well known as defensive secretions of true bugs (e.g., Aldrich et al., 1999; see also El-Sayed, 2014; Ho and Millar, 2002; Oelschlägel et al., 2015). It has been demonstrated that some kleptoparasitic flies feed on injured true bugs, and find this food source using the defensive secretions as kairomones (Kondo et al., 2011; Oelschlägel et al., 2015; Zhang and Aldrich, 2004). As described for deceptive *Aristolochia rotunda* (Oelschlägel et al., 2015), *C. cycniflora* most likely lures its kleptoparasitic milichiid pollinators by mimicking harmed Heteroptera.

For *C. haygarthii* and *C. nilotica* the evidence for potential models is less clear. The main scent component of *C. nilotica*, i.e., ethyl dodecanoate, however, has been found in pheromones of various hymenopterans (Coppée et al., 2008; Kullenberg et al., 1970; Leonhardt et al., 2009). In case these hymenopterans are used as food sources by the pollinating flies of *C. nilotica*, a kleptomyiophilous pollination strategy can be considered.

Ceropegia carnosa, in contrast, is pollinated by Phoridae of the genus *Megaselia*. The ecology of adult phorid flies, including *Megaselia*, is highly diverse. Parasitoids (Dutto and Ferrazzi, 2014; Gonzalez et al., 2002; Menail et al., 2016), specialist predators, saprophages and even kleptoparasites are found in this genus (Disney, 1994; Disney and Fayle, 2008; Hash, 2014). Floral scent of *C. carnosa* contained the common green leaf volatiles (*E*)-2-hexenal and (*Z*)-3-hexenyl acetate

(e.g., Ruther, 2000). Species of *Megaselia* act as parasitoids of several insects, including herbivores (Zvereva and Rank, 2004), and it is well known that parasitoids use green leaf volatiles to find their hosts (e.g., Paré and Tumlinson, 1999; Whitman and Eller, 1990). (*E*)-2-Hexenal and (*Z*)-3-hexenyl acetate are further described to occur in secretions of true bugs (Aldrich et al., 1993; see also El-Sayed, 2014; Marques et al., 2000; Sachin et al., 2008), which are also parasitized by *Megaselia* (Costa et al., 2007). This information allows speculating that *C. carnosa* mimics an oviposition site for its phorid pollinators.

Ceropegia rupicola and *C. crassifolia* are pollinated by drosophilid flies, which feed on and breed in different kinds of (over)ripe fermenting fruit (Walsh et al., 2011). Yeast-produced chemicals are associated with fermentation processes (Magee and Kosaric, 1987) and released from fermenting fruits (Stökl et al., 2010) which are used by Drosophilidae as food sources and oviposition sites (Becher et al., 2012). The most prominent of such fermentation volatiles, i.e., acetoin, 2,3-butanediol, and 2,3-butanedione, are the main floral volatiles of *C. crassifolia*. It seems likely that *C. crassifolia* targets its drosophilid pollinators through olfactory mimicry of fermentation, a strategy also used by a deceptive *Arum* species (Stökl et al., 2010). Mimicry of fermenting fruits is also possible for *C. rupicola*, since its floral scent contained isobutyl acetate and 3-methyl-1-butanol. These volatiles are released from different ripe fruits (Jordán et al., 2001; Shalit et al., 2001; Zabetakis and Holden, 1997) and biologically active in *Drosophila* (Becher et al., 2012; Schubert et al., 2014).

Ceropegia stenantha is pollinated by Scatopsidae, and the main floral components are benzaldehyde, a widespread floral volatile (Knudsen et al., 2006), and 1-phenyl-2,3-butanedione, a rather uncommon floral volatile (Joulain, 1987; Wong and Teng, 1994). Some minor identified scent components of *C. stenantha* were not known from nature before (see above) and synthesized for the first time. Bioassays with these floral components were not successful yet, and the association between floral scent components and the biology of pollinating scatopsid flies is still to be revealed. Scatopsidae, commonly called scavenger flies, are described as detritivores (Freeman, 1985; Haenni, 1997; Haenni and Vaillant, 1994) and nectar/pollen feeders (García-Robledo and Mora, 2007; Larson et al., 2001; Woodcock et al., 2014). It remains unclear how *C. stenantha* attracts its scatopsid pollinators and which behavior of these flies is exploited.

Flowers of *Ceropegia barklyi*, *C. pachystelma*, and *C. woodii* predominantly emit hydrocarbons (i.e., decane, heptadecenes) and are pollinated by ceratopogonids of the genus *Forcipomyia*. These flies use leaf-litter as egg-laying substrate (Winder and Silva, 1972), and adults have (klepto)parasitic habits, which include sucking blood from vertebrates and arthropods (Downes, 1958; Gepp, 1982; Glukhova, 1989; Marshall et al., 2015; Martens et al., 2008). Available data on floral scent do not allow any speculations on how *C. barklyi*, *C. pachystelma*, and *C. woodii* trick *Forcipomyia* into non-rewarded pollination.

This lack of definite evidence is also true for *C. ampliata*, the only species pollinated by big sized calyptrate flies (see above, and Coombs et al., 2011). Whether the main floral volatile methyl phenylacetate or other scent components are key in attracting the flies to the flowers of *C. ampliata* remains subject of future studies. *C. ampliata* does produce minute quantities of nectar (Coombs et al., 2011) and has a noticeable wide flower tube (see Figure 1). It can be suggested that this species probably represents a shift to the rewarding open flowers of *Brachystelma*, which are also pollinated by flies bigger than 3 mm (pers. obs., Figure 6). *Brachystelma* is phylogenetically nested in *Ceropegia*, and recent phylogenetic analyses indeed revealed that *C. ampliata* is sister to a subclade mostly containing *Brachystelma* species (see Bruyns et al., 2015).

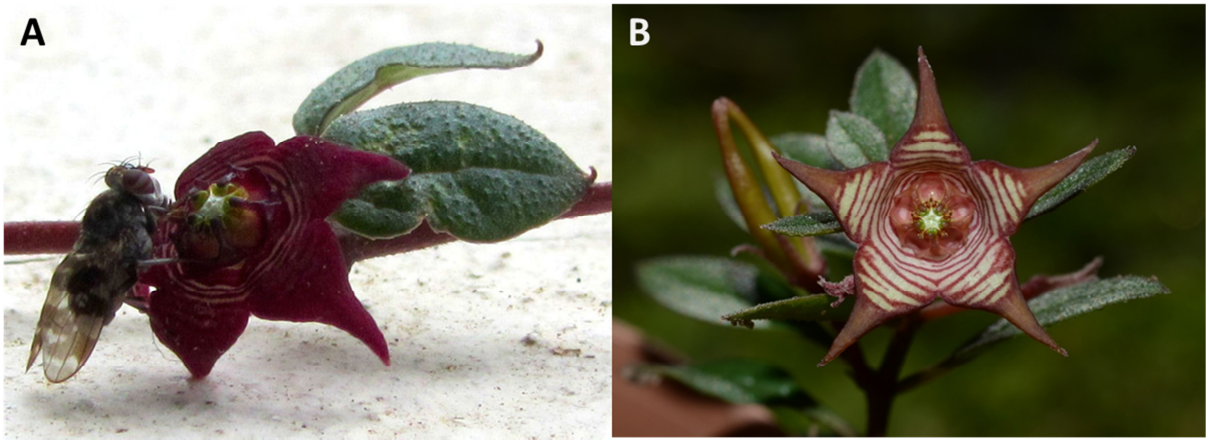


Figure 6: Flower of **A:** *Brachystelma pulchellum* with *Cestrotus* sp. (Lauxaniidae) fly pollinator, and **B:** *B. modestum*. Photographs: A. Heiduk.

Conclusions

My PhD project represents the first comparative and multifaceted study on the pollination system of *Ceropegia*. I gathered comprehensive new data on the chemical ecology and pollination biology of *Ceropegia* trap flowers, and I augmented the existing knowledge about flower visiting and pollinating flies with taxa not known to be associated with *Ceropegia* and other trap flowers before. My analyses on floral scent chemistry and its significance for pollinator attraction are pioneer work not only for *Ceropegia* but for myiophilous trap flowers in general. The occurrence of phylogenetic signal in pollinators but not in floral scent is of great value to understand the evolutionary context of the specialized flower-fly relationships in *Ceropegia*. The uncovered congruence in deceptive strategies (i.e., kleptomyiophily, brood-site mimicry) with trap flowers of *Aristolochia* is significant to understand the evolution of deceptive myiophilous trap flowers as such. Despite the large amount of data I gathered, there are several gaps to be filled in further studies. For the majority of investigated species, the attractive scent components and the mimicry strategies remain unknown. As the present study predominantly included South African *Ceropegia* species, a comparative study on Asian species would be of high importance to see whether chemical ecology and pollination biology differ between the distribution centers of *Ceropegia*. A special focus on *C. ampliata*, which differs from other species in floral traits and pollinating fly taxa (flies >3 mm), could reveal how and why there was a shift to rewarding non-trap flowers within *Ceropegia*. The loss of deceptive trap flowers occurred several times in Africa as well as in Asia. A comparative study that includes rewarding open-flowering species of *Brachystelma* could help to shed more light on the evolution of myiophilous pollination systems and the mechanisms for species formation in the megadiverse Ceropegieae.

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PART II - Publications

Included publications

Published:

- **Heiduk, A., Kong, H., Brake, I., von Tschirnhaus, M., Tolasch, T., Tröger, A.G., Wittenberg, E., Francke, W., Meve, U., & Dötterl, S. (2015):** Deceptive *Ceropegia dolichophylla* fools its kleptoparasitic fly pollinators with exceptional floral scent. Frontiers in Ecology and Evolution 3: 66. doi: 10.3389/fevo.2015.00066. (PART II, Publication 1)

- **Heiduk, A., Brake, I., von Tschirnhaus, M., Göhl, M., Jürgens, A., Johnson, Steven D., Meve, U., & Dötterl, S. (2016):** *Ceropegia sandersonii* mimics attacked honeybees to attract kleptoparasitic flies for pollination. Current Biology 26, 2787-2793. (PART II, Publication 2)

- **Heiduk, A., Brake, I., von Tschirnhaus, M., Haenni, J.-P., Miller, R., Hash, J., Prieto-Benítez, S., Jürgens, A., Johnson, Steven D., Schulz, S., Liede-Schumann, S., Meve, U., & Dötterl, S. (2017):** Floral scent and pollinators of *Ceropegia* trap flowers. Flora 232, 169-182 (invited contribution). (PART II, Publication 3)

Publication 1: Deceptive *Ceropegia dolichophylla* fools its kleptoparasitic fly pollinators with exceptional floral scent

Published 2015 in *Frontiers in Ecology and Evolution*

frontiers in Ecology and Evolution

ORIGINAL RESEARCH
published: 03 July 2015
doi: 10.3389/fevo.2015.00066

Deceptive *Ceropegia dolichophylla* fools its kleptoparasitic fly pollinators with exceptional floral scent

*Annemarie Heiduk[1,2], Hanghui Kong[3], Irina Brake[4], Michael von Tschirnhaus[5], Till Tolasch[6], Armin G. Tröger[7], Elisabeth Wittenberg[7], Wittko Francke[7], Ulrich Meve[2] and Stefan Dötterl[1]**

[1] Plant Ecology, Department of Ecology and Evolution, University of Salzburg, Salzburg, Austria, [2] Department of Plant Systematics, University of Bayreuth, Bayreuth, Germany, [3] Key Laboratory of Plant Resources Conservation and Sustainable Utilization, South China Botanical Garden, Chinese Academy of Sciences, Guangzhou, China, [4] Department of Life Sciences, Natural History Museum, London, UK, [5] Department of Biology, University of Bielefeld, Bielefeld, Germany, [6] Animal Ecology 220c, Institute of Zoology, University of Hohenheim, Stuttgart, Germany, [7] Organic Chemistry, University of Hamburg, Hamburg, Germany

OPEN ACCESS

Edited by:
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Reviewed by:
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***Correspondence:**
Stefan Dötterl, Department of Ecology and Evolution, Plant Ecology, University of Salzburg, Hellbrunnerstr. 34, 5020 Salzburg, Austria
stefan.doetterl@sbg.ac.at

Specialty section:
This article was submitted to Chemical Ecology, a section of the journal Frontiers in Ecology and Evolution

Received: *30 March 2015*
Accepted: *10 June 2015*
Published: *03 July 2015*

Citation:
Heiduk A, Kong H, Brake I, von Tschirnhaus M, Tolasch T, Tröger AG, Wittenberg E, Francke W, Meve U and Dötterl S (2015) Deceptive Ceropegia dolichophylla fools its kleptoparasitic fly pollinators with exceptional floral scent. Front. Ecol. Evol. 3:66. doi: 10.3389/fevo.2015.00066

Ceropegia species (Apocynaceae) have deceptive pitfall flowers and exploit small flies as pollinators, supposedly by chemical mimicry. Only preliminary data on the composition of flower scents are available for a single species so far, and the mimicry system is not yet understood in any species. We collected data on basic pollination aspects of *C. dolichophylla*, analyzed floral scent by gas chromatography linked to mass spectrometry (GC/MS), identified electrophysiologically active scent components by gas chromatography coupled with electroantennographic detection (GC/EAD), and determined compounds responsible for pollinator attraction in bioassays. We found that flowers of *C. dolichophylla* are visited by small flies of several taxa. Only Milichiidae and Chloropidae carried pollinaria and are, thus, pollinators. The pollen transfer efficiency (PTE) at two different sites was 2% and 4%, respectively. The floral scent was dominated by spiroacetals, mainly (2*S*,6*R*,8*S*)-8-methyl-2-propyl-1,7-dioxaspiro[5.5]undecane, n-tridecane, and N-(3-methylbutyl)acetamide. This spiroacetal and the acetamide elicited the most intense electrophysiological responses in fly antennae, and bioassays confirmed the capability of the spiroacetal in eliciting behavioral responses in pollinators. Most flies, determined as pollinators of *C. dolichophylla,* are kleptoparasites. They exploit insect prey of predatory arthropods as food source to which they are attracted by volatiles. 8-Methyl-2-propyl-1,7-dioxaspiro[5.5]undecane and N-(3-methylbutyl)acetamide have not been identified before as volatiles of other plants, however, they are known as insect volatiles. Both compounds occur in the venom glands of paper wasps, a potential food source for the pollinators of *C. dolichophylla*. We propose that *C. dolichophylla* shows a kleptomyiophilous pollination strategy. It mimics insect related odors to exploit the food-seeking behavior of its kleptoparasitic pollinators.

Keywords: fly pollination, kleptomyiophily, kleptoparasites, spiroacetals, food deception

Introduction

Apart from the showiness of visual cues, flowers often use floral scent to attract their pollinators (Raguso, 2008). Floral scents usually advertise a food source provided by the flowers. In deceptive plants, however, flower scents are a false promise of a reward, such as food, a mating partner or an oviposition site (Salzmann et al., 2007; Jürgens et al., 2013; Bohmann et al., 2014) that these plants do not actually offer. Among these cheaters are plants of the genus *Ceropegia* L. (Apocynaceae, Asclepiadoideae) with more than 200 described species, characterized by sophisticated pitfall flowers (Vogel, 1961; Masinde, 2004). Despite great morphological diversification, the basic floral structure is similar among these species (see Vogel, 1961), and the functionality of the pollination system is extremely specialized and conservative (Vogel, 1961; Ollerton et al., 2009). Species investigated so far are pollinated by small flies (but see Coombs et al., 2011), which are trapped inside the flowers for a limited time during which they deposit or take up pollinaria/pollinia. The fly pollinators of *Ceropegia* belong to diverse families, but, typically, only species of a single or a few fly families interact with a single species of *Ceropegia* (Ollerton et al., 2009). This specificity is likely due to distinct floral scents, which are responsible for pollinator attraction (Vogel, 1961; Heiduk et al., 2010). It was suggested that the flowers mimic rotting plant material, male sex pheromones or animal related odors, leading to the idea that pollinating flies are attracted by chemical deceit (Vogel, 1961; Ollerton et al., 2009). In a preliminary analysis on *C. dolichophylla* Schltr., Heiduk et al. (2010) proposed that this species mimics a food-source for its pollinators. *C. dolichophylla* and other *Ceropegia* species are pollinated by kleptoparasitic flies, which are known to feed on the insect prey of predatory arthropods that they find on the basis of volatile insect secretions (Robinson and Robinson, 1977; Sivinski and Stowe, 1980; Sabrosky, 1983; Sivinski, 1985; Eisner et al., 1991; Sivinski et al., 1999). Heiduk et al. (2010) showed the natural scent of *C. dolichophylla* to be highly attractive to flies, however, the compounds attracting the pollinators could not be identified. Furthermore, the study was based on greenhouse grown plants of *C. dolichophylla* and was not conducted in Asia, where it is native. Its natural pollinators were still unknown, and other pollination aspects such as pollination success have not yet been studied in *C. dolichophylla*. Also, the composition of floral scent of wild plants and its attractiveness to fly pollinators in the natural habitat was not determined.

The aim of the present study was to collect additional data on basic pollination aspects for *C. dolichophylla* in its native range in China and to identify flower volatiles that mediate the pollination system. We specifically asked: (1) Who are the natural pollinators? (2) What is the pollination success in natural populations? (3) Which compounds characterize the floral scent of wild (two different areas) and greenhouse plants? (4) Is the natural flower bouquet attractive to pollinators? (5) Which scent components can be perceived by the pollinators? (6) Do specific electrophysiologically active volatiles attract pollinators?

Thus, we collected and identified flower visitors in the native range, and determined the pollen transfer efficiency (PTE) of plants in natural habitats. We also investigated the floral scent of wild plants in China using dynamic headspace methods followed by gas chromatography linked to mass spectrometry (GC/MS). We tested the pollinator attractiveness of natural flower scent and identified electrophysiologically and behaviorally active compounds by gas chromatography coupled with electroantennographic detection (GC/EAD) and field bioassays, respectively.

Abbreviations: PTE, pollen transfer efficiency; GC/MS, gas chromatography linked to mass spectrometry; GC/EAD, gas chromatography coupled with electroantennographic detection.

Materials and Methods

Plant Species

Ceropegia dolichophylla Schtlr. is a climbing herb that grows in forests from 500 to 1500 m a.s.l. Typically, it twines on other vegetation up to 1.5 m in height. Anthesis of individual flowers lasts for 1 day (opening in the morning; Heiduk et al., 2010), and the flowering season spans from July to early September (Zhou and Xie, pers. comm.; eFloras, 2015). Fruits can be found beginning at the end of September (Zhou and Xie, pers. comm.). In *Ceropegia*, the pollen is packed into discrete packages, the pollinia, two of which are connected via caudicles and the corpusculum to form a pollinarium. Due to the complicated pollination mechanism with fused and highly synorganized reproductive organs (gynostegium), where pollinia of a previously extracted pollinarium need to be inserted between "guide rails" (Vogel, 1961), *C. dolichophylla* depends on pollinators for successful reproduction.

Study Sites

Investigations were conducted in both China and Germany. In July and August 2012 bioassays were performed at the non-native location in Bayreuth, Germany, where previous studies took place on greenhouse plants (Heiduk et al., 2010). In the native range, plants of *C. dolichophylla* were studied in the Mt. Fanjing area in northeast Guizhou province, China (27°49′N-27°50′N, 108°44′E-108°46′E). This area bears vegetation characterized by broad-leaved evergreen forests of high diversity, in a subtropical, humid monsoon climate. *C. dolichophylla* plants were studied at two sites (henceforth *Area 1* and *Area 2*) approximately 5 km apart from each other. At both sites, floral scent, flower visitors/pollinators, and data on pollen transfer efficiency (PTE) were collected in September 2013. Bioassays were performed in August 2012 and September 2013 at *Area 1* and *Area 2* and additionally in South China Botanical Garden (SCBG), Guangzhou (distance to *Area* 1 and *Area* 2: ca. 1000 km), where *C. dolichophylla* does not naturally occur.

Voucher specimens collected in China were deposited in the herbarium of the University of Bayreuth [Voucher/Accession: China: Guizhou, Tongren, Fanjing Mt., 874 m, A. Heiduk, I. Schäffler and Y. Hong, Sep. 2012 (UBT)].

Flower Visitors/Pollinators and Pollen Transfer Efficiency (PTE)

To obtain information about pollination of *C. dolichophylla* in its native range in China, ca. 400 flowers from 12 individual plants (*Area 1*: 5 plants; daily collection from 7 to 13th September 2013; *Area 2*: 7 plants; collection on 8th September 2013) were picked in the evening and checked for trapped insects. Insects trapped inside the flowers were examined for pollinaria and number of pollinia carried, and only those carrying pollinaria/pollinia were designated as pollinators. To determine pollination success, 267 flowers from *Area 1* and 58 flowers from *Area 2* were examined in the field using a 10× hand lens, and the gynostegia were checked for pollinaria removal and pollinia insertion. For both areas, the mean number of removed pollinaria as well as the mean number of inserted pollinia was calculated. These data were used to calculate PTE separately for *Area 1* and *Area 2*. PTE was calculated as the percentage of removed pollinia that were inserted between guide rails. Since each pollinarium consists of two pollinia, the mean number of inserted pollinia was divided by twice the mean number of removed pollinaria (Johnson et al., 2005; Coombs et al., 2009, 2011).

Collection of Volatiles

Floral volatiles were collected during daytime from newly opened flowers *in situ* using dynamic headspace methods (Dötterl et al., 2005b). Flowers were enclosed in a polyester oven bag (6 × 5 cm; Toppits®, Germany) for 10 min to allow accumulation of floral scent. Subsequently, volatiles were trapped by pulling the air from the bag through small adsorbent tubes (Varian Inc. ChromatoProbe quartz microvials; length: 15 mm, inner diameter: 2 mm) for 5 min using a membrane pump (G12/01 EB, Rietschle Thomas Inc., Puchheim, Germany; flow rate: 200 ml/min). The tubes contained 1.5 mg Tenax-TA (mesh 60–80) and 1.5 mg Carbotrap B (mesh 20–40; both Supelco) fixed by glass wool plugs.

In *Area 1* seven samples were collected from seven different plants. Five of the samples were collected from a single flower, one sample was collected from two flowers, and one from three flowers (in cases where flowers grew closely together, they were enclosed in a single bag to avoid any injury of flowers). In *Area 2* seven samples were collected from five plants (from two of these plants, two samples each were collected). Four of the samples were collected from single flowers, two samples from two flowers, and one sample from three flowers. At each location samples of the surrounding air were also collected as controls.

To obtain solutions of natural scent (19 in total) for bioassays and electrophysiological analyses (see below), floral scent from 17 individual flowers (*Area 1*, Fanjing Mt., China), and two individual flowers (Bayreuth, Germany) was collected for at least 4 h into large adsorbent tubes (glass capillaries; length: 8 cm, inner diameter: 2.5 mm) containing 15 mg Tenax-TA (mesh 60–80) and 15 mg Carbotrap B (mesh 20–40). The trapped volatiles were eluted with 70 µl of acetone (SupraSolv, Merck KgaA, Germany; following Dötterl et al., 2005a) per adsorbent tube. Subsequently, 2×5 and 1×7 samples collected in China, and both samples collected in Bayreuth, were combined to provide three samples (2 × 350 µl, 1 × 490 µl) from field plants (China) and one sample (140 µl) from greenhouse plants (Bayreuth), for further experiments (see below).

Chemical Analysis

The volatiles trapped in small adsorbent tubes were analyzed by GC/MS using an automatic thermal desorption (TD) system (TD-20, Shimadzu, Japan) coupled to a Shimadzu GC/MS-QP2010 Ultra equipped with a ZB-5 fused silica column (5% phenyl polysiloxane; 60 m, i.d. 0.25 mm, film thickness 0.25 µm, Phenomenex). The samples were run with a split ratio of 1:1 and a constant helium carrier gas flow of 1.5 ml/min. The GC oven temperature started at 40°C, then increased by 6°C/min to 250°C and held for 1 min. The MS interface worked at 250°C. Mass spectra were taken at 70 eV (EI mode) from m/z 30 to 350. GC/MS data were processed using the GCMSolution package, Version 2.72 (Shimadzu Corporation 2012).

The solvated scent samples were analyzed by GC/MS using a Shimadzu GCMS-QP2010 Ultra equipped with an AOC-20i auto injector (Shimadzu, Tokyo, Japan) and again a ZB-5 fused silica column (5% phenyl polysiloxane; 30 m long, inner diameter 0.32 mm, film thickness 0.25 µm, Phenomenex). One µl of the samples was injected (injection temperature: 220°C; split ratio: 1:1), and the column flow (carrier gas: helium) was set at 3 ml/min. The GC oven temperature was held at 40°C for 1 min, then increased by 10°C/min to 220°C and held for 2 min. The MS interface worked at 220°C. Mass spectra were again taken at 70 eV (in EI mode) from m/z 30 to 350 and data processed as described above.

Identification of the compounds was carried out using the NIST 11, Wiley 9, FFNSC 2, Adams (2007) databases, the database available in MassFinder 3, and published plotted spectra (Francke et al., 1981; Bergström et al., 1982; Francke and Kitching, 2001). Structures of several compounds were confirmed by comparing mass spectra and retention times with those of synthetic reference samples. The assignment of 8-methyl-2-propyl-1,7-dioxaspiro[5.5]undec-3-ene, was based on the mass spectrum of the natural product and the general fragmentation pattern of spiroacetals (Francke and Kitching, 2001).

Double bond positions of alkenes were determined by reaction with dimethyl disulfide (DMDS) (Buser et al., 1983) and subsequent separation of the adducts on a 30 m × 0.25 mm i.d. 0.25 µm film thickness HP5-MS fused silica capillary column (Agilent Technologies, Santa Clara, CA, USA), starting at 60°C for 3 min, increased at a rate of 3°C/min to 300°C, held for 70 min.

Total scent emission was estimated by injecting known amounts of monoterpenoids, aromatics, and aliphatics (added to small adsorbent tubes). The mean response of these compounds (mean peak area) was used to determine the total amount of each compound extracted from the small adsorbent tubes (Dötterl et al., 2005b).

Statistical Analysis

To screen for quantitative differences in absolute amounts of scent between *Area 1* and *Area 2*, the total amount of scent

per sample and flower was compared between areas using a *t*-test (StatSoft Inc., 2005). Normality was tested by Shapiro-Wilk test and homogeneity of variances by Hartley's test (StatSoft Inc., 2005).

To screen for semi-quantitative (percentage amount contributed per compound) differences in scent among samples of plants from *Area 1* and plants from *Area 2*, the Bray-Curtis (BC) similarity index was calculated using Primer 6.1.11, including the add-on package Permanova + 1.0.1 (Clarke and Gorley, 2006; Anderson et al., 2008). If more than one sample was taken from the same individual plant, the mean scent composition was calculated and used for analyses. Based on the BC matrix a PERMANOVA (Factor: *Area*; 10,000 permutations) was performed using the same software package to test for an *Area* effect.

Electrophysiological Analysis

The scent components from *C. dolichophylla* flowers that were perceived by flower visitors/pollinators, were identified by gas chromatography coupled to electroantennographic detection (GC/EAD) and GC/MS (see above). Altogether 34 GC/EAD measurements with 14 flies from China and seven measurements with five flies from Bayreuth, Germany, were performed. The five flies (female *Desmometopa sordida*) from Bayreuth were collected from flowers of *C. dolichophylla* greenhouse plants. Four (one *Oscinella frit*, one *Desmometopa varipalpis*, two *Neophyllomyza* sp.) of the 14 Chinese flies were collected at SCBG while feeding on dead honey bees. The other 10 flies were attracted during bioassays performed in China with synthetic compounds of the *C. dolichophylla* flower scent (see bioassays). All flies were kept separately in Eppendorf® tubes (1.5 ml) with a piece of humid paper towel and stored in the dark at 4°C until electrophysiological measurements were performed.

For measurements, the head of a fly was cut off at the base of the thorax, mounted between two electrodes filled with insect Ringer's solution (8.0 g/l NaCl, 0.4 g/l KCl, 0.4 g/l $CaCl_2$) and connected to silver wires. The reference electrode was placed in contact with the cutting surface of the head while the recording electrode was brought into contact with the tip of the funiculus (cf. first flagellomere) of an antenna.

For measurements we either used a Carlo Erba Vega 6000 Series 2 (Rodano, Italy) or an Agilent 7890A (Santa Clara, California, USA) gas chromatograph, both equipped with a flame ionization detector (FID) and an EAD setup (heated transfer line, 2-channel USB acquisition controller) provided by Syntech (Kirchzarten, Germany). For each measurement 1 µl of an acetone solution of the *C. dolichophylla* scent was injected (injector temperature at 250°C) in splitless mode at 40°C oven temperature. The oven of both systems was heated at a rate of 10°C/min to 220°C, and the split vent was opened 0.5 min after injection. A Zebron ZB-5 column was used for analysis (5% phenyl polysiloxane; 30 m × 0.32 mm i.d. film thickness 0.25 µm, Phenomenex) in both GCs. The column of the Carlo Erba GC was split at the end by the four-arm flow splitter GRAPHPACK 3D/2 (Gerstel, Mühlheim, Germany) into two deactivated capillaries (length 50 cm × 0.32 mm i.d.) leading to the FID and to the EAD setup. Nitrogen was introduced as a make-up gas through the fourth arm of the splitter. The column of the Agilent GC was split at the end by a µFlow splitter (Gerstel, Mühlheim, Germany) into two deactivated capillaries leading to the FID (2 m × 0.15 mm i.d.) and EAD (1 m × 0.2 mm i.d.) setup. In both systems the outlet of the EAD was placed in a cleaned and humidified airflow directed over the fly antenna. Acetone solutions of the scent of *C. dolichophylla* were tested on antennae of five female *D. sordida* (3 × 1 and 2 × 2 runs per specimen), one female *D.* sp. nr. *sordida* (1 × 3 runs), two female *D. varipalpis* (5 and 3 runs), six female *Neophyllomyza* sp. (2 × 1, 2 × 2, and 2 × 3 runs), three female *N. leanderi* (1, 2, and 3 runs), one female *Conioscinella* sp. (2 runs), and one female *Oscinella frit* (3 runs). After the measurements, head and body of each fly were stored in a 4% solution of glycerin in ethanol (99.8%) for identification of genus and/or species.

Synthesis of EAD-active Compounds

Racemic spiroacetals were synthesized according to established methods (Phillips et al., 1980; Jacobsen et al., 1982; Doubský et al., 2004). To prepare (2*S*,6*R*,8*S*)-8-methyl-2-propyl-1,7-dioxaspiro[5.5]undecane (**S4a**) (**Figure 1**), commercially available 2-ethoxycarbonylcyclopentanone (**a**) was alkylated to yield the disubstituted cyclopentanone **b**, which after acidic hydrolysis was decarboxylated to produce 2-propylcyclopentanone (**c**). Baeyer-Villiger oxidation of **c** produced the racemic lactone **d**. Alkynylation of (2*S*)-2-methyloxirane (**e**) using lithium acetylide yielded (2*S*)-4-pentyne-2-ol (**f**) which was benzylated to **g**. Racemic **d** and the anion of **g** were linked to form the intermediate **h** (not isolated) which upon hydrogenation furnished the (8*S*)-configured spiroacetal **S4** as a mixture of the three stereoisomers **S4a-c** (see **Figure 2A**).

To a solution of 2.67 g (15.4 mmol) (*S*)-benzyloxypent-4-yne (**g**) in 30 mL abs. THF, cooled to −78°C, were dropwise added 7.50 mL (18.8 mmol) of a 2.5 M-solution of n-BuLi in hexane. After stirring for 90 min at −78°C, 2.00 mL (16.2 mmol) $BF_3 \bullet Et_2O$, dissolved in 20 mL abs. THF, were slowly added, followed by a solution of 2.39 g (16.8 mmol) 6-propyltetrahydro-2*H*-pyran-2-one (**d**) in 10 mL THF. Over a period of 3 h, the mixture was warmed to room temperature, and the reaction was quenched by the addition of a mixture of 20 ml water, 20 mL diethyl ether, and ammonium chloride/ammonia (2:1). After separation of the layers, the aqueous phase was extracted 4 times with 20 mL portions of diethyl ether. The combined organic solutions were washed with brine and dried over magnesium sulfate. Filtration over silica and removal of the solvent *in vacuo* yielded 4.35 g of crude **h**. This was dissolved in 10 mL methanol and hydrogenated for 21 h at 20 bar, using 5% Pd-C catalyst. After removal of the catalyst by filtration over silica, the crude product (see **Figure 2A**) was purified by chromatography on silica using a 50:1-mixture of pentane and diethyl ether. A further chromatographic step using benzene as the eluent yielded 113 mg (0.53 mmol, 3.5%) highly pure **S4a** (see **Figure 2B**).

NMR-Spectra were run on a Bruker (Billerica, MA, USA) AMX-400 instrument. For the numbering of structural elements see **Figure 2B**.

^{1}H-NMR, based on **^{1}H-^{1}H-COSY, HSQC, HMBC** (400 MHz, $CDCl_3$): **δ** [ppm] = 0.92 (t, $^3J_{H14\text{-}H13}$ = 7.1 Hz, 3H, CH_3 C14),

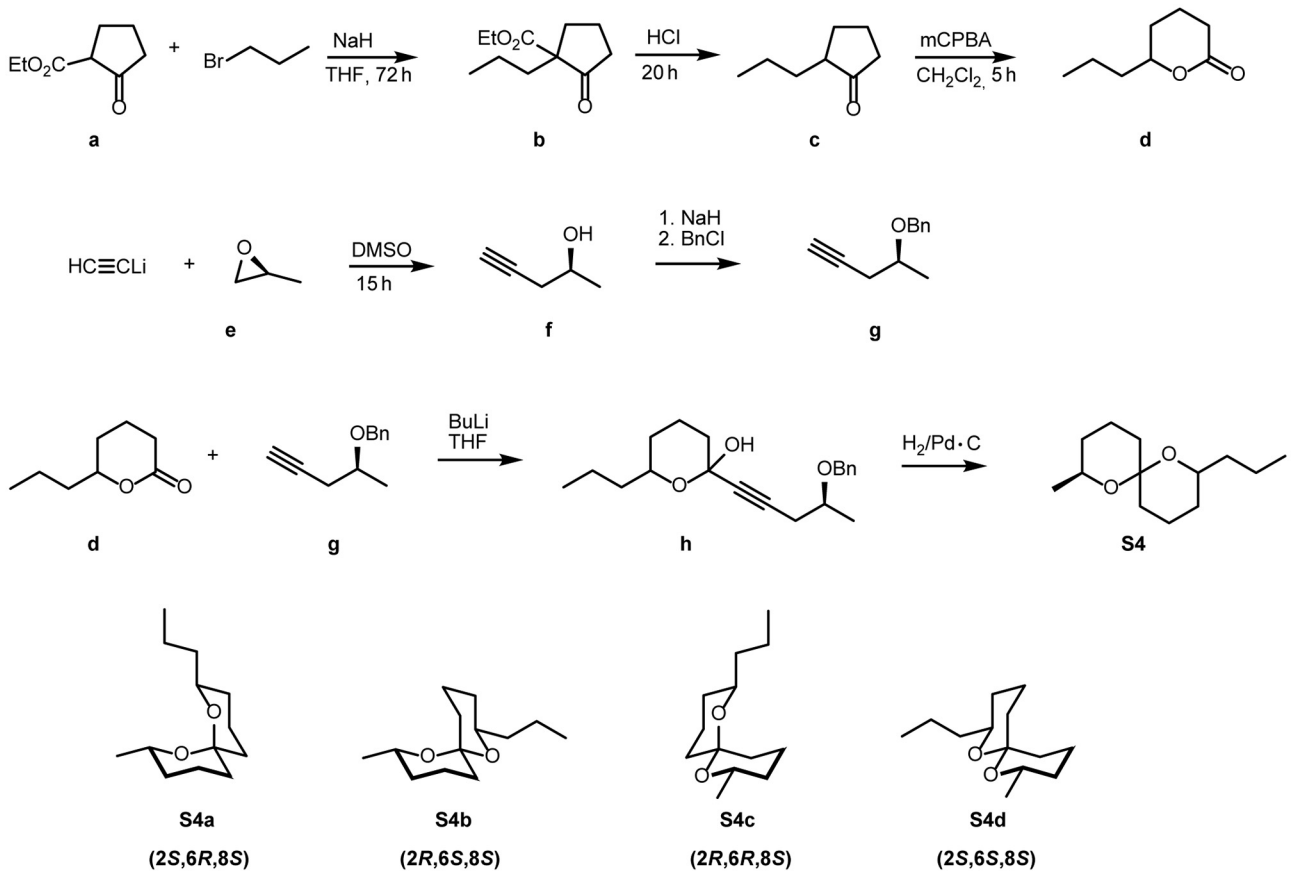

FIGURE 1 | Synthesis of (8*S*)-8-methyl-2-propyldioxaspiro[5.5]undecanes S4a–d.

1.10–1.20/1.51–1.59 (2m, 2H, CH_2 C3 ax/eq), 1.12–1.23/1.54–1.61 (2m, 2H, CH_2 C9 ax/eq), 1.13 (d, $^3J_{H15\text{-}H8} = 6.3$ Hz, 3H, CH_3 C15), 1.30–1.39/1.46–1.55 (2m, 2H, CH_2 C13), 1.33–1.43/1.56–1.64 (2m, 4H, CH_2 C5 C11 ax/eq), 1.31–1.40/1.43–1.51 (2m, 2H, CH_2 C12), 1.50–1.58/1.88 (m/ddddd, $^3J_{H-H} = 13.9, 13.2, 13.2, 4.0, 4.0$ Hz, 4H, 2 × CH_2 C4 C10 eq/ax), 3.54 (dddd, $^3J_{H-H} = 11.0, 8.7, 4.0, 2.0$ Hz, 1H, CH C2ax), 3.70 (dqd, $^3J_{H-H} = 11.4, 6.3, 2.0$ Hz, 1H, CH C8ax).

^{13}C-NMR, based on **HSQC, HMBC** (126 MHz, $CDCl_3$): **δ** [ppm] = 14.39 (q, C14), 19.09 (t, C10), 19.16 (t, C13), 19.28 (t, C4), 21.99 (q, C15), 31.55 (t, C3), 33.01 (t, C9), 35.50/35.69 (2t, C5 C11), 38.89 (t, C12), 65.21 (d, C8), 68.85 (d, C2), 96.12 (s, C6).

The 70 eV mass spectrum of **S4a** was identical to the plotted one published earlier (Francke et al., 1981).

Due to the double anomeric effect (Deslongchamps et al., 1981) and the equatorial orientation of both alkyl substituents (Francke et al., 1980), **S4a** was the highly dominating stereoisomer. The two thermodynamically less stable (*E*,*Z*)-isomers **S4b** and **S4c** were formed as by-products, whereas the diequatorially linked highly unstable **4d** was not obtained in detectable amounts. The same synthetic approach, but using racemic 2-methyloxirane yielded a mixture of all eight possible stereoisomers of 8-methyl-2-propyl-1,7-dioxaspiro[5.5]undecane (rac-**S4**), dominated by the racemate of the (2*E*,8*E*)-isomer. Enantioselective gas chromatography, employing a home-made 30 m × 0.25 mm i.d. fused silica capillary coated with a 1:1-mixture of OV-1701 and heptakis-[2,3-di-*O*-methyl-6-*O*-(*tert*-butyldimethylsilyl)]-β-cyclodextrin as the stationary phase, separated the enantiomers well. Hydrogen as the carrier gas at a constant oven temperature of 90°C produced an α-value of 1.22 = (ret. time 2*S*,6*R*,8*S*):(ret. time 2*R*,6*S*,8*R*).

Bioassays

The attractiveness of acetone solutions of the scent of *C. dolichophylla* (see above) was tested in China (for experiments in Germany see Heiduk et al., 2010). Samples were assayed on six different days (4× Fanjing Mt. *Area 1*, 2× SCBG). Each time a glass vial containing an acetone solution of natural scent (see before) was offered against a similar glass vial filled with pure acetone (control). Within a distance of 30 cm to each other the vials were tucked into the ground and offered for at least 30 min and up to 60 min. The amount of scent available in a sample was sufficient for two assays. The attractiveness of single EAD-active compounds was tested both in China (Fanjing Mt., SCBG) and in Germany (Bayreuth). The compounds were chosen based on preliminary assays with fractions of the complete flower scent. We used

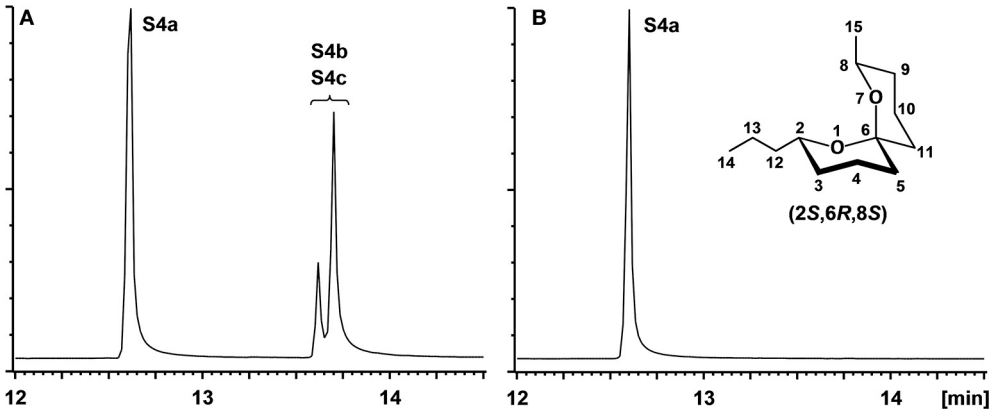

FIGURE 2 | (A) Gas chromatogram of crude **S4a** containing small amounts of the three stereoisomers; **(B)** Gas chromatogram of purified **S4a**.

the major EAD-active (see below) volatile compounds N-(3-methylbutyl)acetamide (**1**), stereochemically pure (2*S*,6*R*,8*S*)-8-methyl-2-propyl-1,7-dioxaspiro[5.5]undecane (**S4a**) and its racemate (including ca. 5% of the three other stereoisomers, which slightly differs from the natural proportions, see 2.8). Racemic (*E*,*E*)-2,8-diethyl-1,7-dioxaspiro[5.5]undecane was tested in addition. Since this compound eluted shortly after **S4a,** it was potentially considered also EAD-active. (*E*,*E*)-8-Methyl-2-propyl-1,7-dioxaspiro[5.5]undecane was also used as a racemic mixture. The substances were diluted (dilution: 10^{-3}; v/v; final volume: 400–600 µl) in acetone (SupraSolv, Merck KgaA, Germany) and offered in glass vials similar to those used for tests with natural samples. As determined by dynamic headspace and GC/MS for **S4a**, the amount of scent released from the vials resembled the amount of scent released from single flowers as described by Heiduk et al. (2010).

We tested (a) the single components, **1**, **S5a,** and **S4/S4a**, (b) the three possible two-component mixtures, (c) the three component mixture, and (d) all three components against each other. When using mixtures, proportions were adapted to the ratios found in *C. dolichophylla* flowers as indicated by dynamic headspace and GC/MS analysis. Vials containing the samples were tucked into the ground with a distance of 20 cm to each other and offered for at least 40 min and up to 60 min. In each bioassay a glass vial with pure acetone was offered as the control. Approaching flies showed a characteristic zig-zag flight with abrupt landing. They were caught when arriving within a maximum distance of 10 cm to the vial containing the sample. Due to their fast and frantic behavior, not all approaching flies could be caught.

Results

Flower Visitors/Pollinators and PTE

The flowers of *C. dolichophylla* collected in China altogether contained 119 dipteran individuals, 107 thereof were collected in *Area 1* and the other 12 in *Area 2*. The flies belonged to the families Milichiidae, Chloropidae, Phoridae, and to taxa of lower Diptera (**Table 1**). Chloropidae were only present in flowers of *Area 1*.

Different taxa from lower Diptera were the most abundant visitors, however, they did not carry pollinaria - nor did the phorid flies. Milichiids were the second most abundant group, and many of these flies carried pollinaria (60.5%). They were determined as *Desmometopa microps* (13 females, 10 males; 12 with pollinaria), *D. varipalpis* (one female), *Neophyllomyza* sp. (9 females, 7 with pollinaria) and *N. leanderi* (10 females, 7 with pollinaria). With seven individuals, chloropid flies were not very abundant, however, four (57%) of them carried pollinaria. Chloropids were determined as *Conioscinella* sp. (2 females, 1 with pollinarium), *Polyodaspis* sp. (1 male, 2 females, all with pollinaria), and *Tricimba* spp. (2 females of different species, both with pollinaria).

Among the 267 flowers analyzed in *Area 1*, 51 pollinaria were found to be removed and 4 pollinia inserted, resulting in a PTE of 4%. The percentage of flowers with removed pollinaria was 13%, and 1% of flowers had pollinia inserted. On average and per flower, 0.19 pollinaria were removed and 0.02 pollinia inserted. Of the 58 flowers collected in *Area 2* altogether 47 pollinaria were removed and 2 pollinia inserted, yielding a PTE of 2%. The percentage of flowers with removed pollinaria was 28%, and 3% of flowers had pollinia inserted. On average 0.81 pollinaria were removed per flower, whereas 0.03 pollinia were inserted.

Flower Scent

In the floral scent of *C. dolichophylla* 53 different components were detected: 14 spiroacetals (40.3%), 6 alkanes (16.8%), 4 alkenes (9.4%), 4 other aliphatics (0.1%), one nitrogen containing compound (7.4%), and 23 unknown compounds (1.4%) (**Table 2**, **Figure 3**). The most abundant scent components were (2*S*,6*R*,8*S*)-8-methyl-2-propyl-1,7-dioxaspiro [5.5]undecane (**S4a**) (27%), tridecane (15%) and N-(3-methylbutyl)acetamide (**1**) (7,4%), contributing 49% to the total scent. Apart from (*E*,*E*)-2,8-diethyl-1,7-dioxaspiro[5.5]undecane (**S5a**) (8%) all other compounds did not exceed 5%.

The total amount of scent per flower (ng/15 min; 10 min accumulation + 5 min sampling) was highly variable and

TABLE 1 | Flies collected from flowers of *Ceropegia dolichophylla* and attracted to natural flower scent or synthetic scent samples during bioassays in China (Fanjing Mt., SCBG) and Germany (Bayreuth). The locations and the number of (successful) bioassays performed are also given. The sex of flies is indicated if known (f, female; m, male). Bold, numbers of collected/attracted individuals of pollinating species.

	Location	# assays (positive)	# caught flies	***Desmometopa microps* (Lamb, 1914)**	*Desmometopa singaporensis* (Kertész, 1899)	*sordida* (Fallén, 1820) *Desmometopa*	*Desmometopa* sp. nr. *sordida*	*Desmometopa varipalpis* (Malloch, 1927)	*Desmometopa* Loew, 1866 sp.	***Neophyllomyza leanderi* (Hendel, 1924)**	***Neophyllomyza* Melander, 1913 sp.**	*Aphanotrigonum trilineatum* (Meigen, 1830)	***Coniosciniella* Duda, 1929 sp.**	***Polyodaspis* Duda, 1933 sp.**	***Tricimba* Lioy, 1864 sp.1**	***Tricimba* sp.2**	Phoridae	other Brachycera	lower Diptera
Fly families				**Milichiidae**									**Chloropidae**				**Others**		
Collected from flowers	Fanjing Mt.			**13f10m**				1f		**10f**	**9f**		**2f**	**2f1m**	**1f**	**1f**	3	1	65
TWO-CHOICE ASSAY: SUBSTANCE/MIXTURE VS. ACETONE																			
Natural scent sample (in acetone)	Fanjing Mt.	4 (0)	0																
	SCBG	2 (2)	12							**5f**	**7f**								
N-(3-methylbutyl)acetamide (**1**)	Fanjing Mt.	5 (1)	1															1	
	SCBG	5 (0)	0																
	Bayreuth	3 (2)	2	**1f**		1f													
(*E*,*E*)-2,8-diethyl-1,7-dioxaspiro[5.5]undecane (**S5a**)	Fanjing Mt.	3 (0)	0																
	SCBG	1 (0)	0																
	Bayreuth	1 (0)	0																
rac-8-methyl-2-propyl-1,7-dioxaspiro[5.5]undecane as a mixture of all 8 stereoisomers (rac-**S4**)	Fanjing Mt.	3 (2)	4	**1m**									**1f**					1	1
	SCBG	4 (0)	0																
	Bayreuth	1 (1)	7	**7f**															
(2*S*,6*R*,8*S*)-8-methyl-2-propyl-1,7-dioxaspiro[5.5]undecane (**S4a**)	Fanjing Mt.	5 (2)	4															3	1
	SCBG	5 (4)	8		4f					**4f**									
	Bayreuth	5 (5)	62	**57f2m**								1m						2	
TWO-CHOICE ASSAY: SUBSTANCE/MIXTURE VS. ACETONE																			
mixture of **S4a+1** (ratio 8:1)	Fanjing Mt.	4 (2)	4						1m									3	
	SCBG	4 (2)	26		12f				3f	**10f**			**1f**						
mixture of **S5a+1** (ratio 3:1)	Fanjing Mt.	3 (0)	0																
	SCBG	1 (1)	2							**2f**									
mixture of **S4a+S5a** (ratio 8:3)	Fanjing Mt.	3 (0)	0																
	SCBG	1 (1)	0																
mixture of **1+S4a+S5a** (ratio 1:8:3)	Fanjing Mt.	4 (0)	0																
FOUR-CHOICE ASSAY:																			
1 vs. **S4a** vs. **S5a** vs. acetone	SCBG	1 (1)	17[a]				1f	2f		**6f**	**7f**		**1f**						

[a]*All individuals attracted to **S4a**.*

TABLE 2 | Volatiles of *Ceropegia dolichophylla* flowers collected from field plants in China (*Area 1* and *Area 2*) and from greenhouse plants in Germany (Heiduk et al., 2010).

	KRI	Area 1	Area 2	Bayreuth*	
		Median (Min–Max) ($N = 7$ plants)	**Median (Min–Max)** ($N = 5$ plants)	**Plant 1**	**Plant 2**
Total amount of scent (ng/15 min*flower)		**32.2 (14.6–66.7)**	**79.8 (16.1–117.0)**	**311.3**	**195.1**
ALIPHATICS					
Alkanes					
Undecane[#1]	1100	0.3 (0–**12.8**)	1.5 (0–**18.0**)	0	0
Tridecane[#1]	1302	**7.0** (1.4–**54.0**)	**22.2** (**13.3**–**40.2**)	**19.7**	**22.3**
2-Acetoxyundecane	1432	tr (0–1.0)	0.3 (0–1.8)	0	0
Pentadecane[#1]	1500	0.3 (0–0.6)	0.4 (0.2–1.1)	0.7	0.8
2-Acetoxytridecane[#2]	1629	0.7 (0.2–**5.1**)	0.8 (0.3–2.6)	2.4	2.1
Alkenes					
6-Tridecene **3**[EAD]	1289	0.1 (0–0.7)	1.1 (0.1–2.2)	0.9	2.8
5-Tridecene **4**[EAD]	1291	0.4 (0–1.7)	1.2 (0.3–2.9)	0.9	1.6
6,9-Pentadecadiene **8**[EAD]	1481	1.9 (0.1–**10.7**)	3.8 (1.1–**9.5**)	4.6	**5.4**
6- + 7-Pentadecene **9**[EAD]	1485	1.8 (0.5–**14.6**)	3.6 (1.4–**18.1**)	**10.6**	**12.4**
5-Pentadecene	1491	0.1 (0–1.0)	2.1 (0–**18.1**)	0.1	0.3
SPIROACETALS					
(*E,E*)-2,8-Dimethyl-1,7-dioxaspiro[5.5]undecane **S1a**[#3,EAD]	1149	2.8 (0.7–**14.3**)	3.1 (0.1–**11.8**)	**7.8**	**10.6**
2-Ethyl-7-methyl-1,6-dioxaspiro[4.5]decane **S2**[#3,EAD]	1163	0 (0–0.4)	0 (0–0.6)	0	0
(*E,Z*)-2,8-Dimethyl-1,7-dioxaspiro[5.5]undecane **S1b**[#3,EAD]	1223	0.1 (0.1–0.4)	0.1 (0–0.7)	0.6	0.7
(*E,E*)-2-Ethyl-8-methyl-1,7-dioxaspiro[5.5]undecane **S3**[#3,EAD]	1239	0.2 (0.1–0.1)	0.7 (0.1–3.2)	0.4	0.8
(2*S*,6*R*,8*S*)-8-Methyl-2-propyl-1,7-dioxaspiro[5.5]undecane **S4a**[#3,EAD]	1325	**33.0** (**9.6**–**59.3**)	**21.8** (**14.5**–**43.6**)	**36.1**	**28.4**
(*E,E*)-2,8-Diethyl-1,7-dioxaspiro[5.5]undecane **S5a**[#3,EAD]	1330	**9.0** (3.2–**16.0**)	**6.5** (4.0–**12.1**)	**10.9**	**7.8**
7-Ethyl-2-propyl-1,6-dioxaspiro[4.5]decane **S6**[EAD]	1333	0.3 (0–0.4)	0.2 (0.1–0.4)	0	0
8-Methyl-2-propyl-1,7-dioxaspiro[5.5]undec-3-ene **S7**[EAD]	1346	0.2 (0.1–0.4)	0.2 (0–0.3)	0.2	0.1
2-Ethyl-7-propyl-1,6-dioxaspiro[4.5]decane **S8**[EAD]	1354	0.1 (0–0.2)	0.1 (0–0.2)	0	0
2-Ethyl-8-methyl-1,7-dioxaspiro[5.6]dodecane **S9**	1377	0.1 (0.1–0.3)	0.1 (0.1–0.2)	0.3	0
(*E,Z*)-2,8,Diethyl-1,7-dioxaspiro[5.5]undecane **S5b**[#3,EAD]	1389	0.3 (0.2–0.6)	0.3 (0.2–0.6)	0.4	0.3
(*E,Z*)/(*Z,E*)-8-Methyl-2-propyl-1,7-dioxaspiro[5.5]undecane **S4b**/**c**[#3,EAD]	1392	0.3 (0.1–0.7)	0.3 (0.1–0.9)	0.5	0.4
(*E,Z*)/(*Z,E*)-8-Methyl-2-propyl-1,7-dioxaspiro[5.5]undecane **S4b**/**c**[#3]	1397	0.3 (0.1–0.7)	0.3 (0.2–0.7)	0.6	0.6
(*Z,Z*)-8-Methyl-2-propyl-1,7-dioxaspiro[5.5]undecane **S4d**[#3]	1449	0.1 (0.1–0.3)	0.1 (0.1–0.2)	0.1	0.1
OTHERS					
Undecan-2-one[#1]	1296	0 (0–0.1)	0.1 (0–0.3)	0	0
α-Ionone	1444	0	0	0.1	0.2
Tridecan-2-one **10**[#1,EAD]	1499	0 (0–0.4)	tr (0–0.2)	0	0
NITROGEN CONTAINING COMPOUNDS					
N-(3-Methylbutyl)acetamide **1**[#3,EAD]	1135	**10.3** (**7.8**–**52.1**)	4.5 (1.2–**11.1**)	1.7	0.8
UNKNOWNS[a]		0.9 (0.3–2.3)[21]	1.2 (0.4–7.8)[21]	0.1[1]	0.1[1]
m/z: 55,97,115 **5**[EAD]	1332	0.3 (0–0.7)	0.1 (0–0.3)	0	0
m/z: 45,83,97,126,154 **11**[EAD]	1504	0 (0–0.3)	tr (0–tr)	0	0

*KRI, Kovats retention index; tr, amount <0.05%; in bold, values >5.0%; [#]Compound verified through authentic standard, which were purchased from Sigma-Aldrich ([#1]) or available in the collections of TT ([#2]) and WF ([#3]); EAD, electrophysiologically active; *Samples collected by Heiduk et al. (2010) and reanalyzed for present work; [a]Upper script digits indicate the number of compounds pooled.*

ranged from 15 to 67 ng in *Area 1* (median: 32 ng), and 16 to 117 ng in *Area 2* (median: 80 ng), respectively. There was no significant difference in total amounts of scent between *Area 1* and *Area 2* [$t_{(10)} = -2.0$, $p = 0.074$]. Furthermore, no significant differences in scent profiles (relative scent composition) were found [PERMANOVA: Pseudo-$F_{(1, 10)}$: 1.4, $p = 0.198$] between plants of *Area 1* and *Area 2*.

A comparison of scent from field and greenhouse (Heiduk et al., 2010) plants revealed eight compounds exclusively present in field plants and two compounds only present in greenhouse plants (**Table 2**).

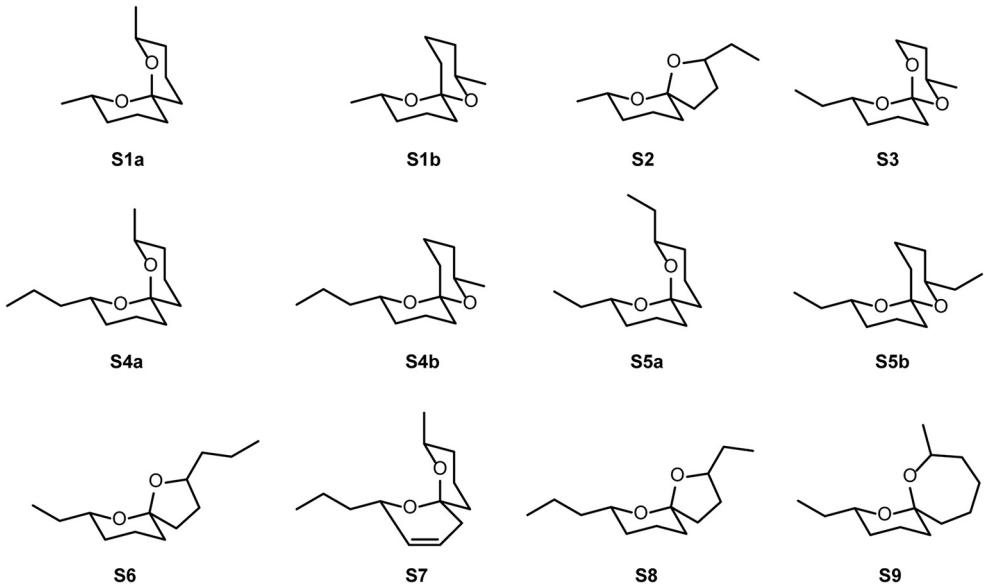

FIGURE 3 | Structures of spiroacetals S1–S9 without stereochemical assignments. For stereochemically correct structures of naturally occurring **S4a–d** see **Figure 1**.

Electrophysiological Analysis

Only two (*Desmometopa* sp. nr. *sordida* and *D. varipalpis*) of the 14 flies from China and three of the five female *D. sordida* from Bayreuth gave obvious antennal signals. Antennae of the other flies had too much noise in their signals and, thus, were not included in the analysis.

Of the 53 components found in scent samples of *C. dolichophylla* collected in the field, 22 compounds (**Table 2**) were electrophysiologically active in the two species tested. The antenna of *D. varipalpis* responded to 19 compounds, most strongly to N-(3-methylbutyl)acetamide (**1**), (2*S*,6*R*,8*S*)-8-methy-2-propyl-1,7-dioxaspiro[5.5]undecane (**S4a**) + (*E*,*E*)-2,8-diethyl-1,7-dioxaspiro[5.5]undecane (**S5a**), (*E*,*Z*)-2,8-diethyl-1,7-dioxaspiro[5.5]undecane (**S5b**), and an unknown compound (**Figure 4A**). Of the 19 components with EAD-activity in *D. varipalpis*, the antenna of *D.* sp. nr. *sordida* responded only to **1** and to 6,9-pentadecadiene (**8**) as well as to 6- + 7-pentadecene (**9**) (**Figure 4A**).

All three female *D. sordida* from Bayreuth responded to the same seven compounds (**Figure 4B**). Five of them were also active on flies from China and scent samples from plants collected in the field. In each run, **1** and/or **S4a** + **S5a** elicited the strongest antennal responses (**Figure 4**).

Bioassays

During bioassays in China (Fanjing Mt. and SCBG) and Germany (Bayreuth) only Diptera were attracted, and no fly responded to the negative controls (**Table 1**).

Samples of natural flower scent were tested in China (for experiments in Germany see Heiduk et al., 2010) at Fanjing Mt. *Area 1* (4 replicates), and at SCBG (2 replicates), and only at SCBG were flies attracted. The first flies approached in zig-zag flight within a minute after opening the sample vial. Altogether, 12 of the approaching flies (seven female *Neophyllomyza* sp., five female *N. leanderi*) were caught, all of them from taxa that occur as natural pollinators of *C. dolichophylla*.

In bioassays with synthetic samples (China and Germany), altogether 137 attracted flies were collected, the majority of them in Bayreuth ($N = 71$) and SCBG ($N = 53$), and a few ($N = 13$) in Fanjing Mt. Their behavior in approaching the sample vials was identical to that elicited by samples of natural flower scent. Milichiidae were the most numerous attracted flies (97%). They represented eight different species, among them three species pollinating *C. dolichophylla*. Two different species of Chloropidae were represented by four flies, and one of these species was identified as a pollinator of *C. dolichophylla*. The remaining flies were from non-pollinating taxa.

Flies were attracted mostly by (2*S*,6*R*,8*S*)-8-methyl-2-propyl-1,7-dioxaspiro[5.5]undecane (**S4a**) and mixtures containing this spiroacetal. Pollinating species were attracted to **S4a**, N-(3-methylbutyl)acetamide (**1**), to the mixture of **S4a** + **1**, and to the mixture of (*E*,*E*)-2,8-diethyl-1,7-dioxaspiro[5.5]undecane **(S5a)** + **1**. Non-pollinating species responded to the same lures except the mixture of **S5a** + **1**.

In the four-choice assays which offered **1**, **S5a**, **S4a**, and acetone, all flies responded to **S4a**. Overall, most flies were attracted to **S4a**, and the majority of them were the pollinating milichiid *D. microps*. The majority of non-pollinating species were attracted to the mixture of **S4a** + **1**.

Discussion

This study specifies milichiid and chloropid flies as pollinators of *C. dolichophylla*, shows that the pollination rate is low,

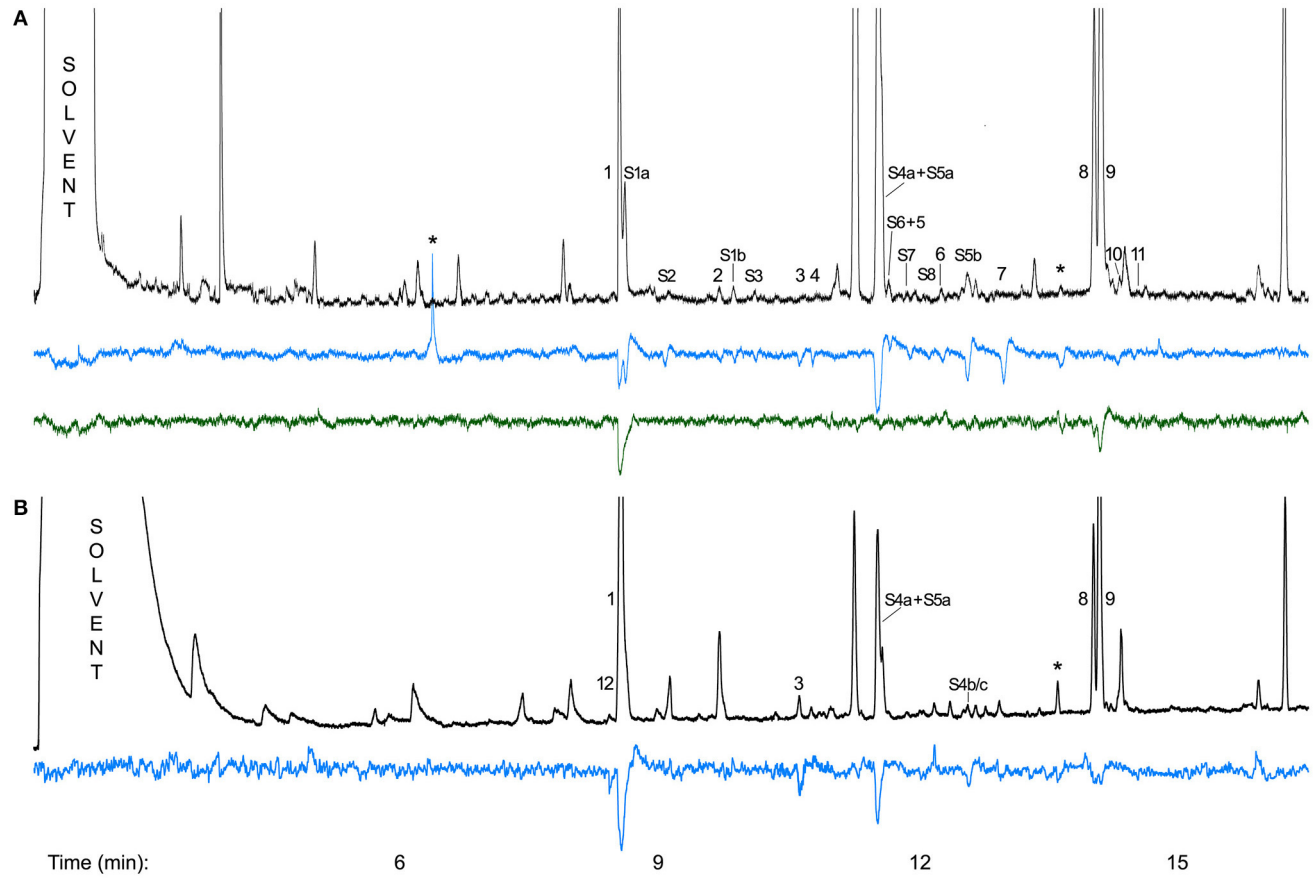

FIGURE 4 | (A) Antennal responses of a female *Desmometopa varipalpis* (blue) and a female *D.* sp. nr. *sordida* (green) to components of the flower scent of *Ceropegia dolichophylla* collected in Fanjing Mt., China; **(B)** Antennal responses of a female *Desmometopa sordida* to a flower scent sample of *C. dolichophylla* collected in Bayreuth, Germany. **1**, N-(3-methylbutyl)acetamide; **S1a**, (*E*,*E*)-2,8-Dimethyl-1,7-dioxaspiro[5.5]undecane; **S2**, 2-Ethyl-7-methyl-1,6-dioxaspiro[4.5]decane; **2**, unknown compound not detected in TD-samples; **S1b**, (*E*,*Z*)-2,8-Dimethyl-1,7-dioxaspiro[5.5]undecane; **S3**, (*E*,*E*)-2-Ethyl-8-methyl-1,7-dioxaspiro[5.5]undecane; **3**, 6-Tridecene; **4**, 5-Tridecene; **S4a + S5a**, (2*S*,6*R*,8*S*)-8-Methy-2-propyl-1,7-dioxaspiro[5.5]undecane + (*E*,*E*)-2,8-diethyl-1,7-dioxaspiro[5.5]undecane; **S6 + 5**, 7-Ethyl-2-propyl-1,6-dioxaspiro[4.5]decane + KI 1332; **S7**, 8-Methyl-2-propyl-1,7-dioxaspiro[5.5]undec-3-ene; **S8**, 2-Ethyl-7-propyl-1,6-dioxaspiro[4.5]decane; **6**, unknown compound not detected in TD-samples; **S5b**, (*E*,*Z*)-2,8-Diethyl-1,7-dioxaspiro[5.5]undecane; **7**, unknown compound not detected in TD-samples; **8**, 6,9-Pentadecadiene; **9**, 6- + 7-Pentadecene; **10**, 2-Tridecanone; **11**, KI 1504; **12**, unknown compound not detected in TD-samples; **S4b/c**, (E,*Z*)/(Z,E)-8-Methyl-2-propyl-1,7-dioxaspiro[5.5]undecane. *, artifact/response to contamination; KI, Kovats retention index. All compounds except **S3** elicited signals in at least two of the three species and/or were consistently active in repeated measurements with single individuals.

and identifies an uncommon spiroacetal, (2*S*,6*R*,8*S*)-8-methyl-2-propyl-1,7-dioxaspiro[5.5]undecane (**S4a**), as the main scent component and as a compound capable of attracting fly pollinators.

Identification of flies trapped in flowers revealed natural pollinators of *C. dolichophylla*. They belong to several milichiid and chloropid genera, only two of which (Milichiidae: *Desmometopa*, *Neophyllomyza*) were previously described as flower visitors of *C. dolichophylla* (Heiduk et al., 2010). All species found to act as pollinators in China (**Table 1**, printed in bold) did not occur as pollinators of *C. dolichophylla* in Germany (Heiduk et al., 2010), and the milichiid fly *D. sordida*, pollinator of *C. dolichophylla* in Germany (Heiduk et al., 2010) does not pollinate the flowers in China. This discrepancy can only partly be explained by the distribution range of the flies, because several of the Chinese pollinators (e.g., *N. leanderi*, *D. microps*) occur in Germany as well. *D. sordida* has been found in Mongolia (Papp, 1976) and Japan (Iwasa, 1996) and is likely to occur in China, though possibly not as far south as our study site. Chloropid flies were not present in flowers of *Area 2*. However, all flowers from this area were sampled on a single day and due to local population dynamics chloropid flies might just have been absent in *Area 2* at that point of time. Furthermore, floral scent as the attractive cue did not differ among the sites and should thus not have been responsible for observed differences in the presence of Chloropidae.

Though the abundance of pollen carrying flies was quite high in flowers, the pollination success was found to be very low

in the investigated species. This finding is consistent with data published for *C. ampliata* (Coombs et al., 2011), the only other *Ceropegia* studied in this context.

The pollinating taxa of *C. dolichophylla* identified to species level are not yet known as visitors/pollinators of other *Ceropegia* species, however, all genera except *Polyodaspis* are already known from *Ceropegia* (Knuth , 1898–1905; Vogel, 1961, 1993; Masinde, 2004; Ollerton et al., 2009; Heiduk et al., 2010). Milichiidae and Chloropidae have rarely been described as pollinators in other angiosperms, but are known as pollinators from other Apocynaceae (Raspi et al., 2009; Pisciotta et al., 2011), rewarding and non-rewarding orchid species (Borba and Semir, 2001; Chase et al., 2014; Nunes et al., 2014), and several species of *Aristolochia* (Brantjes, 1980; Wolda and Sabrosky, 1986; Oelschlägel et al., 2015). Lower Diptera were the most abundant flower visitors but did not carry pollinia and, therefore, are no pollinators of *C. dolichophylla*. However, different taxa of lower Diptera are described as pollinators for several other *Ceropegia* species (Ollerton et al., 2009). Lower Diptera are small enough to enter the flowers of *C. dolichophylla* but they fail as pollinators probably due to morphological features. Successful removal of pollinaria requires an optimal fit of the fly headfirst into the coronal cavities below and around the guide rail entrances. After insertion of the proboscis (or parts of it) the fly has to be strong enough to pull the pollinarium off the style-head. Possibly, the proboscides of lower Diptera are too short for successful guide rail insertion or pollinarium attachment, or the flies are too weak to remove the pollinarium. Selection against flies that are either too big or too small through morphological features is also described in *Aristolochia*, another plant group with pitfall flowers pollinated by flies (Berjano et al., 2009; Oelschlägel et al., 2009).

As shown already by Heiduk et al. (2010) and confirmed in the present study, flower visiting/pollinating flies are attracted to extracts of natural scent samples. We additionally identified corresponding biologically active compounds.

Our electrophysiological studies show that only a subset of the volatiles, including most of the spiroacetals, is perceived by the flies (**Table 2**, **Figures 4A,B**). Furthermore, we found that there are differences in perception among different pollinating fly species. Nevertheless, all tested species perceive at least one of the main compounds N-(3-methylbutyl)acetamide (**1**) or (2*S*,6*R*,8*S*)-8-methyl-2-propyl-1,7-dioxaspiro[5.5]undecane (**S4a**). Furthermore, in field bioassays **S4a** was especially attractive to flies of several taxa, including pollinators. This spiroacetal as well as the other spiroacetals identified in the present study are unknown plant volatiles (cf. Knudsen et al., 2006). Generally, spiroacetals are rare constituents of floral scent. Just recently spiroacetals were shown to have a function in attracting pollinators, as they are key signals for host plant recognition of a solitary bee that specializes on *Campanula* flowers (Milet-Pinheiro et al., 2013).

Despite being rare in floral scents, spiroacetals are very widespread in nature and also produced by microorganisms and animals, including mammals. However, the biological significance of these compounds is known only in a few cases (Francke and Kitching, 2001). Apart from a few exceptions, the carbon skeletons are unbranched and show an uneven number of carbon atoms.

Consistent with Heiduk et al. (2010), we found in bioassays that flies respond very quickly, mostly within the first minute after being offered the test sample. This underlines the outstanding importance of the *C. dolichophylla* floral scent in attracting fly pollinators. The quick response of the flies could also explain why within the natural population of *C. dolichophylla* only low numbers of flies were attracted. *C. dolichophylla* flowers open in the morning shortly before sunrise (Heiduk et al., 2010), and bioassays were performed only after sunrise. Thus, most flies available in the habitat may already have been trapped by newly opened flowers before bioassays took place.

Several of the flies attracted by flowers of *C. dolichophylla* (e.g., Milichiidae: *Desmometopa, Neophyllomyza*; Chloropidae: *Conioscinella*, *Tricimba*) are kleptoparasites which feed on preyed-upon insects (Frost, 1913; Robinson and Robinson, 1977; Sivinski and Stowe, 1980; Landau and Gaylor, 1987; Eisner et al., 1991; Sivinski et al., 1999; Zhang and Aldrich, 2004; Marshall, 2012; Von Tschirnhaus et al., 2014), such as wasps, bees, lacewings, and true bugs. Interestingly, secretions of such insects contain compounds identified as biologically active scent compounds of *C. dolichophylla* in the present study. Among them are several spiroacetals, N-(3-methylbutyl)acetamide (**1**), 6-tridecene (**3**), 7-pentadecene (**9**), and 2-tridecanone (**10**) (Dani et al., 2000; Francke and Kitching, 2001; Bruschini et al., 2006; El-Sayed, 2014). Venom glands of paper wasps (*Polistes*), for example, contain 8-methyl-2-propyl-1,7-dioxaspiro[5.5]undecane (**S4**), (*E*,*E*)-2,8-dimethyl-1,7-dioxaspiro[5.5]undecane (**S1a**), 2-ethyl-7-methyl-1,6-dioxaspiro[4.5]decane (**S2**), and N-(3-methylbutyl)acetamide (**1**) (see Bruschini et al., 2006). Both spiroacetals **S1a** and **S2** have also been identified in the cephalic secretions of *Andrena* bees (Francke et al., 1981; Bergström et al., 1982). Interestingly, (*E*,*E*)-2,8-dimethyl-1,7-dioxaspiro[5.5]undecane keeps (2*S*,6*R*,8*S*)-configuration in *A. wilkella* (Tengö et al., 1990)—the same stereochemistry as in the major spiroacetal of *C. dolichophylla*. Thus, volatile signals and constituents of defense glands of bees and/or wasps could well be mimicked by *C. dolichophylla*. Indeed, preyed upon wasps fighting against an arthropod predator (e.g., praying mantis, spider) are a food source for kleptoparasitic flies (Micallef, 2010). Moreover, wasps are predators themselves, and the flies may seek for the wasps' prey item. Wasps stun and/or kill their prey using their venom, and kleptoparasitic flies might use these venom volatiles as key signals to locate a wasp with fresh prey, on which they could feed. Therefore, *C. dolichophylla* probably makes use of compounds which indicate the presence of prey items for food-seeking kleptoparasitic flies. Thus, the flowers are kleptomyiophilous and fool kleptoparasitic flies into pollinating them.

Kleptomyiophily was unknown until recently, when it was discovered in *Aristolochia* and *Ceropegia* in parallel. Oelschlägel et al. (2015) described it for the first time for a deceptive *Aristolochia* species pollinated by kleptoparasitic Chloropidae. Independently from each other, the early diverged lineage *Aristolochia* (39.5 million years ago; Naumann et al., 2013) and the much younger group *Ceropegia* (10 million years ago; Rapini et al., 2007) evolved both, the trap flowers and the kleptomyiophilous pollination strategy.

To conclude, we show that deceptive *C. dolichophylla* fools its kleptoparasitic fly pollinators by a kleptomyiophilous pollination strategy using exceptional floral scent. Flowers emitted several spiroacetals, many of which were known from insect secretions, but unknown in floral scents before this study. Additional compounds released were N-(3-methylbutyl)acetamide (**1**) and aliphatic alkenes. Several of the compounds elicited electrophysiological responses in antennae of fly pollinators, among them (2*S*,6*R*,8*S*)-8-methyl-2-propyl-1,7-dioxaspiro[5.5]undecane (**S4a**). This spiroacetal was proven to be highly attractive for pollinators in behavioral assays. Further studies will show whether other *Ceropegia* species also evolved a kleptomyiophilous pollination strategy and if so, which compounds they use to trick their pollinators.

Author Contributions

AH, SD, and UM designed the study. HK performed bioassays in China in 2012. AH collected all other field data. SD performed the electrophysiological measurements. IB and MV identified fly pollinators. WF, AT, EW, and TT identified and/or synthesized compounds. AH analyzed the data and wrote the first draft of the manuscript. WF wrote the part of the chemical synthesis. All authors contributed to interpretation of the findings and edited and approved the manuscript.

Acknowledgments

The authors thank Irmgard Schäffler, Yu Hong, Yun Zhou, and Xiaolin Xie for help during field trips, and Irmgard Schäffler additionally for help with preparation of Figures. We also thank Frank Menzel for assistance with the identification of flies, and Kjirsten Wayman for valuable comments on the manuscript. This research was supported by a grant for PhD candidates according to Bavarian elite promotion law (BayEFG) and Natural Science Foundation of China (Project No. 31470319, 30900088).

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publication 2: *Ceropegia sandersonii* mimics attacked honeybees to attract kleptoparasitic flies for pollination

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Ceropegia sandersonii Mimics Attacked Honeybees to Attract Kleptoparasitic Flies for Pollination

Highlights

- *Ceropegia sandersonii* flowers lure and trap carnivorous flies for pollination
- The flowers emit volatiles also released by honeybees signaling distress
- Pollinating flies use such volatiles to locate bees under attack as a food source
- The flowers are a chemical mimic of a food source of adult carnivorous flies

Authors

Annemarie Heiduk, Irina Brake, Michael von Tschirnhaus, ..., Steven D. Johnson, Ulrich Meve, Stefan Dötterl

Correspondence

stefan.doetterl@sbg.ac.at

In Brief

Heiduk et al. describe a new pollination strategy in plants. They show that trap flowers of *Ceropegia sandersonii* mimic alarm substances of honeybees to lure food-stealing fly pollinators, which use bees under attack as a food source.

Heiduk et al., 2016, Current Biology *26*, 2787–2793
October 24, 2016
http://dx.doi.org/10.1016/j.cub.2016.07.085

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Ceropegia sandersonii Mimics Attacked Honeybees to Attract Kleptoparasitic Flies for Pollination

Annemarie Heiduk,[1,7] Irina Brake,[2] Michael von Tschirnhaus,[3] Matthias Göhl,[4] Andreas Jürgens,[5,6] Steven D. Johnson,[6] Ulrich Meve,[7] and Stefan Dötterl[1,8,*]
[1]Department of Ecology and Evolution, Plant Ecology, University of Salzburg, 5020 Salzburg, Austria
[2]Department of Life Sciences, Natural History Museum, London SW7 5BD, United Kingdom
[3]Department of Biology, University of Bielefeld, 33615 Bielefeld, Germany
[4]Department of Organic Chemistry, University of Bayreuth, 95440 Bayreuth, Germany
[5]Department of Biology, Plant Chemical Ecology, Technische Universität Darmstadt, 64287 Darmstadt, Germany
[6]School of Life Sciences, University of KwaZulu-Natal, Pietermaritzburg, Private Bag X01, Scottsville 3209, South Africa
[7]Department of Plant Systematics, University of Bayreuth, 95440 Bayreuth, Germany
[8]Lead Contact
*Correspondence: stefan.doetterl@sbg.ac.at
http://dx.doi.org/10.1016/j.cub.2016.07.085

SUMMARY

Four to six percent of plants, distributed over different angiosperm families, entice pollinators by deception [1]. In these systems, chemical mimicry is often used as an efficient way to exploit the olfactory preferences of animals for the purpose of attracting them as pollinators [2,3]. Here, we report a very specific type of chemical mimicry of a food source. *Ceropegia sandersonii* (Apocynaceae), a deceptive South African plant with pitfall flowers, mimics attacked honeybees. We identified kleptoparasitic *Desmometopa* flies (Milichiidae) as the main pollinators of *C. sandersonii.* These flies are well known to feed on honeybees that are eaten by spiders, which we thus predicted as the model chemically mimicked by the plant. Indeed, we found that the floral scent of *C. sandersonii* is comparable to volatiles released from honeybees when under simulated attack. Moreover, many of these shared compounds elicited physiological responses in antennae of pollinating *Desmometopa* flies. A mixture of four compounds—geraniol, 2-heptanone, 2-nonanol, and (*E*)-2-octen-1-yl acetate—was highly attractive to the flies. We conclude that *C. sandersonii* is specialized on kleptoparasitic fly pollinators by deploying volatiles linked to the flies' food source, i.e., attacked and/or freshly killed honeybees. The blend of compounds emitted by *C. sandersonii* is unusual among flowering plants and lures kleptoparasitic flies into the trap flowers. This study describes a new example of how a plant can achieve pollination through chemical mimicry of the food sources of adult carnivorous animals.

RESULTS AND DISCUSSION

One of the most species-rich genera (~200 species; [4]) with deceitful trap flowers is the fly-pollinated genus *Ceropegia* L. (Apocynaceae, Asclepiadoideae). Among fly families that pollinate *Ceropegia* [5, 6] are taxa with kleptoparasitic habits, which steal food from other animals, i.e., predatory arthropods (e.g., spiders), by feeding on hemolymph or other secretions released by the predators' prey items [7–12]. It is generally believed that kleptoparasitic flies find such food sources by volatile organic compounds released by the prey items after a predator attack, possibly in combination with factors generated by the predator [7, 11, 13–15]. This information suggests that kleptoparasitic flies mistake the flower scent of *Ceropegia* for the odor of a food source (*C. dolichophylla* [16, 17]), as recently discovered in an *Aristolochia* species [18]. However, whether these plants chemically mimic a specific model remains unknown.

Here we elucidated the pollination biology, floral scent, and chemical mimicry system in *C. sandersonii*, known as Giant Ceropegia. Our specific objectives were to (1) determine which of the flower visitors are pollinators and whether pollinators are kleptoparasitic Diptera, (2) compare floral odor with volatiles of a potential model mimicked by the flowers, (3) identify shared biologically active components by analytical chemistry and electrophysiological methods, and (4) test four candidate compounds involved in pollinator attraction by bioassays in the field.

Flower visitors belonged to various dipteran families, but only Chloropidae and Milichiidae carried pollinaria (Table 1). Flower visitors from other families were smaller and more fragile (e.g., Cecidomyiidae) and might be too weak to remove pollinaria from the flowers (cf. [5, 17]). The majority of visitors trapped in the flowers were different species of *Desmometopa* (Milichiidae). A total of 26% thereof, all from the most abundant species, carried pollinaria (cf. Figure S1). Since species of *Desmometopa* are similar in body size and structure of their mouthparts, to which the pollinaria are attached, we assume that all flower-visiting species are potential pollinators of *C. sandersonii*. *Desmometopa* has a global distribution, consists of more than 50 species

Table 1. Flies Trapped in Flowers of *Ceropegia sandersonii* and Attracted to Synthetic Scent Mixtures

	Flower Visitors and Pollinators		Two-Choice Assay (n = 9): Complete Mixture versus Acetone[a]	Five-Choice Assay (n = 8): Depleted Mixtures versus Acetone				
	Europe	South Africa		A	B	C	D	Ac
Total Number of Flies	70	54	50	258	44	23	30	1
Milichiidae	69	45	44	249	29	12	19	
Desmometopa inaurata Lamb, 1914		5		3				
Desmometopa interfrontalis Sabrosky, 1965				62	3		4	
Desmometopa microps Lamb, 1914†	6(3PolI)							
Desmometopa m-nigrum (Zetterstedt, 1848)		3						
Desmometopa cf. *nudigena* Sabrosky, 1983†		29(16PolI)		37				
Desmometopa sordida (Fallén, 1820)†	6 M, 57(10PolI)	1						
Desmometopa Loew, 1866 indet.(7 morphospecies)		3	37	127	7	2	9	
Enigmilichia Deeming, 1981 sp.				1			1	
Leptometopa rufifrons Becker, 1903		3	5	19	16	3	3	
Milichiella Giglio-Tos, 1895 indet.(5 morphospecies)		1	2		3	7	2	
Chloropidae		3	3	2	10	6	3	
Arcuator Sabrosky, 1985 sp.†		1M[PolI]						
Aphanotrigonum Duda, 1932 sp.							1	
Conioscinella minutissima Séguy, 1938					7	1		
Lasiochaeta Corti, 1909 sp.					1			
Oscinella Becker, 1978 sp.					1			
Rhodesiella infumata (Becker, 1913)					1			
Trachysiphonella Enderlein, 1936 sp. nov.		2	3 M	1 M, 1		5	2 M	
Other Diptera (Cecidomyiidae[F], Ceratopogonidae[F], Chironomidae[F], Dolichopodidae, Fanniidae, Muscidae, Phoridae[F], Platypezidae, Scatopsidae[F], Sciaridae, unknown)	1	6	3	7	5	5	8	1

Number and identity of flies collected from *C. sandersonii* flowers in Europe and South Africa, and flies attracted to bioassays performed in South Africa with mixtures of synthetic scent compounds. Complete mixture contained geraniol, 2-heptanone, 2-nonanol, (*E*)-2-octen-1-yl acetate. Depleted mixtures contained complete mixture without (*E*)-2-octen-1-yl acetate (A), 2-heptanone (B), geraniol (C), or 2-nonanol (D). Species indicated with a dagger symbol (†) are pollinating species. All flies were female unless otherwise indicated. M, male; [PolI], flies with pollinaria; [F], flower-visiting families within Other Diptera. See also Figure S1.

[a]No flies responded to the acetone control.

[19], and has, as do members of other trapped fly groups, a kleptoparasitic habit. This feeding habit is mainly used by female flies to obtain protein for egg production [11], and accordingly, mainly female flies were found in flowers of *C. sandersonii*. *Desmometopa* species have strong preference for honeybees, and the females can frequently be observed feeding in great numbers on fluids of honeybees caught by spiders (Figure 1B; [20–23]).

If it is the case that *C. sandersonii* chemically mimics food sources to deceive *Desmometopa*, we predict that the flowers will emit unusual compounds or specific blends of compounds not normally found in flowers. Furthermore, these compounds are expected to overlap with the volatiles released by honeybees when under attack. Flower scent of *C. sandersonii* is complex and contains widespread and uncommon compounds [24, 25] in a qualitative blend unique among flowering plants. We found that 60% of the floral compounds, including all main compounds, overlapped with the volatiles released from both European and South African honeybee subspecies under simulated

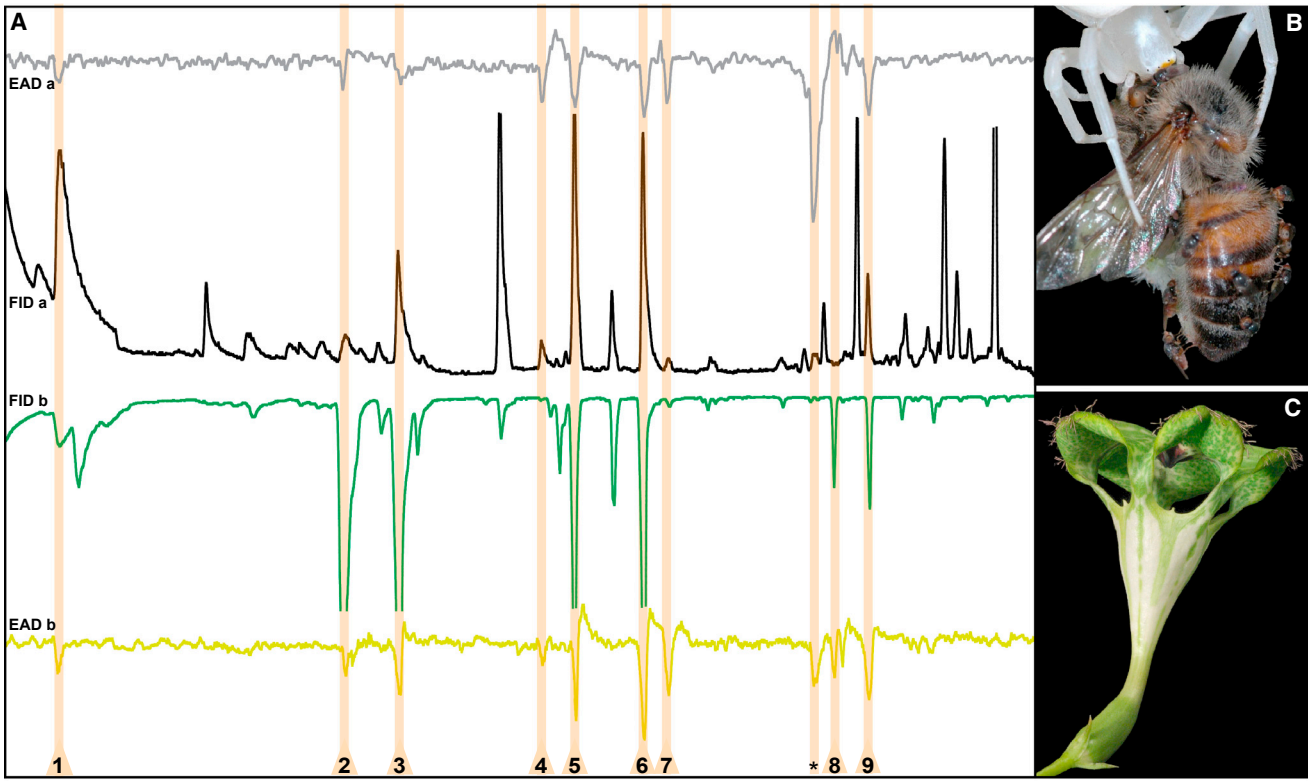

Figure 1. Electrophysiological Measurements and Key Players of Studied Interaction

(A) Examples of antennal responses of female *Desmometopa sordida* (EAD a) to components of honeybees under simulated attack (FID a) and female *D. sordida* (EAD b) to flower scent of *Ceropegia sandersonii* (FID b). EAD-active compounds present in both flowers and bees were **1**: 2-heptanone, **2**: (*E*)-2-octen-1-ol, **3**: 2-nonanol, **4**: hexyl butyrate, **5**: (*E*)-2-octen-1-yl acetate, **6**: geraniol, **7**: geranial, **8**: geranyl acetate, and **9**: (*E*)-2-decen-1-yl acetate. *: unknown compound.

(B) Honeybee being eaten by a spider, with kleptoparasitic flies feeding on fluids leaking from the bee.

(C) *C. sandersonii* flower.

See also Table S1 and FIGURE S2.

attack (Table 2; Table S1; see also [25–33]). We observed a high variation in the relative amount of scent among replicate flower and bee samples (Table 2; Table S1; Figure 1A). This finding points to a high intraspecific variability in the ratio of compounds released from both flowers of *C. sandersonii* and honeybees under simulated attack.

Many of these compounds common in flower and bee samples are known to be produced in mandible glands (e.g., 2-heptanone; [34]) or sting glands (e.g., alcohols and acetate esters; [29, 35–37]) of worker bees and are released from the glands during defensive bites or when a honeybee extrudes its stinger for defense. Due to its anesthetic effects, 2-heptanone is also used by honeybees for defense against arthropods that are too small to be attacked with the stinger [38]. Geraniol, geranial, and neral, which were also present in both flower and honeybee samples, are known to be released from the Nasonov gland [39, 40]. The Nasonov pheromone is known to play an important role in communication among worker bees ([40] and references therein). A defensive role for the Nasonov scent has not been reported before; however, we observed that honeybees caught by spiders and those under simulated attack in the lab both exposed their Nasonov gland. In other *Apis* species, i.e., *A. dorsata* (Fabricius 1793) [41] and *A. nuluensis* Tingek, Koeniger & Koeniger, 1996 [42], the Nasonov pheromone is known to be involved in colony defense behavior, suggesting that a defensive use is also likely in *A. mellifera*.

The high overlap in volatiles between flowers and honeybees supports our hypothesis of food source mimicry in *C. sandersonii*, while our electrophysiological measurements and behavioral assays provide further support. Nearly half of the overlapping compounds were detected by the antennae of *Desmometopa* flies (Table 2; see also Figure 1A), and four of these electrophysiologically active compounds were also behaviorally active. Geraniol, 2-heptanone, 2-nonanol, and (*E*)-2-octen-1-yl acetate attracted kleptoparasitic milichiid and chloropid flies when offered as a 1:1:1:1 mixture ($\chi^2 = 5.59$, $p = 0.018$). The mean ± SE number of flies attracted to the mixture per trial was 5.55 ± 1.44, whereas no insects were attracted to the acetone controls. We selected these four compounds for the bioassays because of their presence in both *C. sandersonii* flower scent and samples of honeybees under simulated attack. Furthermore, preliminary scent and electroantennographic detection (EAD) data indicated that these substances are abundant in flowers (Figure 1A) and elicit strong antennal responses. Owing to the high intraspecific variation, we used these

Table 2. Volatiles Identified in Both Flowers of *Ceropegia sandersonii* and Honeybees under Simulated Attack, and Their Electrophysiological Activity in Fly Pollinators

	Ceropegia sandersonii Flowers		*Apis mellifera* under Simulated Attack		EAD Activity in *Desmometopa*		
	Bayreuth Mean ± SD (n = 2)	BG-UKZN Mean ± SD (n = 3)	*A. m. carnica/ ligustica* Mean ± SD (n = 4)	*A. m. scutellata* Mean ± SD (n = 5)	*D. microps*	*D. sordida*	
					Flower Scent	Flower Scent	Honeybee
Contribution to total scent (%)	92	99	42	60	(n = 16/18)	(n = 6/6)	(n = 2/2)
Aliphatic Compounds							
2-Heptanone[S,MG]†	*20 ± 4*	*27 ± 17*	*7 ± 12*	*9 ± 17*	+++	++	+++
2-Heptanol[S,SG]†	*8 ± 2*	*9 ± 1*	1 ± 2	3 ± 2	++		++
Isobutyl butyrate	tr ± tr	-	-	tr ± tr			
(*Z*)-3-Hexen-1-yl acetate[S]	*6 ± 1*	-	tr ± tr	tr ± tr			
Hexyl acetate[S,SG]	1 ± tr	1 ± tr	3 ± 2	*5 ± 2*			
(*E*)-2-Hexen-1-yl acetate	tr ± tr	-	1 ± tr	1 ± tr			
2-Heptyl acetate[SG]	-	tr ± tr	-	1 ± tr			
(*E*/*Z*)-3-Octen-1-ol	1 ± tr	1 ± tr	1 ± tr	1 ± 1			
(*E*)-2-Octenal	2 ± 1	tr ± tr	-	tr ± tr			
(*E*)-2-Octen-1-ol†	4 ± 1	2 ± 2	1 ± tr	1 ± tr	+++	++	++
1-Octanol[S,SG]	2 ± tr	1 ± tr	1 ± tr	2 ± 1			
2-Nonanone[S]†	3 ± tr	4 ± 1	tr ± tr	1 ± tr	++	+++	++
2-Nonanol[S,SG]†	*12 ± 4*	*33 ± 15*	*5 ± 2*	*20 ± 7*	+++	+++	+++
Hexyl butyrate[S]†	tr ± tr	-	tr ± tr	tr ± tr		+	+++
(*E*/*Z*)-3-Octen-1-yl acetate†	1 ± tr	tr ± tr	tr ± tr	tr ± tr	+++	+	++
(*E*)-2-Octen-1-yl acetate[S,SG]†	*15 ± 5*	2 ± 1	*5 ± 4*	tr ± tr	+++	+++	+++
2-Acetoxynonane†	2 ± 1	*17 ± 6*	1 ± tr	3 ± 1	+++	++	++
(*E*)-2-Decen-1-ol	-	tr ± tr	tr ± tr	tr ± tr			
2-Undecanone[S]†	tr ± tr	tr ± tr	tr ± tr	tr ± tr	++	++	+++
2-Undecanol[S,SG]	-	tr ± tr	tr ± tr	tr ± tr			
(*E*)-2-Octen-1-yl butyrate†	tr ± tr	-	tr ± tr	-	+++	+	
(*E*)-2-Decen-1-yl acetate[SG]†	1 ± tr	tr ± tr	1 ± tr	1 ± tr	+++	+++	+++
2-Acetoxyundecane†	1 ± tr	-	-	tr ± tr	+		
Aromatic Compounds							
Benzyl acetate[S,SG]†	tr ± tr	tr ± tr	*6 ± 4*	3 ± 2	+	+	
C5-Branched Chain Compounds							
3-Methyl-2-buten-1-yl acetate[S,SG]	1 ± tr	1 ± tr	1 ± 1	2 ± tr			
Nitrogen-Containing Compounds							
Indole[S]	1 ± tr	-	tr ± tr	-			
Terpenoids							
(*E*)-β-Ocimene[S]	2 ± tr	-	tr ± tr	tr ± tr			
Linalool[S]	*5 ± 2*	-	tr ± 1	-			
Nerol[S,NG]	tr ± tr	-	-	tr ± tr			
Neral[S,NG]	tr ± tr	-	tr ± tr	tr ± tr			
Geraniol[S,NG]†	3 ± 2	1 ± 1	*8 ± 9*	*6 ± 12*	+++	+++	+++
Geranial[S,NG]†	1 ± tr	-	tr ± tr	tr ± tr	+++	+++	+++
Geranyl acetate[S,NG]†	tr ± tr	tr ± tr	tr ± tr	-	++	+++	+++

Relative amount of volatiles collected from flowers of *C. sandersonii* in South Africa (BG-UKZN) and Germany (Bayreuth UBT) and from honeybees under simulated attack (for a complete list of compounds, see Table S1). [S], compound identification verified through authentic standard. Origin of compounds from various honeybee glands (from [25–27]) is indicated as: [SG], sting gland; [MG], mandible gland; [NG], Nasonov gland. tr, trace amount < 0.5%; values 5.0% or greater are italicized. Electrophysiologically active compounds are indicated by dagger symbols (†), and their activity in antennae of pollinating *Desmometopa microps* and *D. sordida* (n values with slashes / indicate the number of fly individuals and the number of antennae used) is also given (+: <25%; ++: 25%–50%; +++: >50% of flies tested). See also Table S1 and Figure S2.

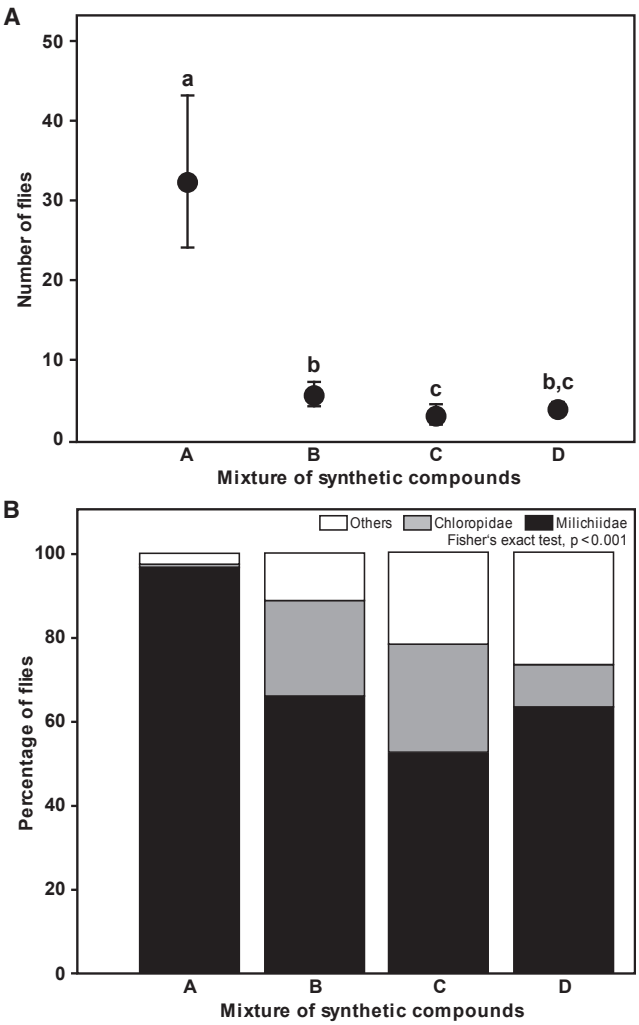

Figure 2. Behavioral Assays with Synthetic Scent Mixtures

Total mean ± SE number (A) and percentage (B) of flies attracted to mixtures of synthetic compounds. Mixtures were offered in five-choice assays (n = 8) together with an acetone control. A: geraniol, 2-heptanone, 2-nonanol; B: geraniol, 2-nonanol, (*E*)-2-octen-1-yl acetate; C: 2-heptanone, 2-nonanol, (*E*)-2-octen-1-yl acetate; D: geraniol, 2-heptanone, (*E*)-2-octen-1-yl acetate.

compounds in a 1:1:1:1 blend (see [43] for a discussion on selecting blends).

The subtractive choice bioassays in which each of one of the four components was omitted from the four blend mixture revealed significant differences among the four three-component mixtures ($\chi^2_{df = 3,\ n = 355} = 39.8$; $p < 0.001$). The mixture depleted of (*E*)-2-octen-1-yl acetate (mix A, Figure 2A) attracted a mean of 32.3 flies and was significantly more attractive than the other mixtures, which all attracted similar small numbers of flies (means 2.8–5.5) (Figure 2A). The overall frequencies of Milichiidae, Chloropidae, and "other Diptera" responding to the bioassays also showed a significant difference among the different mixtures ($p < 0.0001$; Figure 2B). The flies attracted to mix A nearly exclusively (97%) belonged to Milichiidae (Figure 2B), including the most abundant pollinator *Desmometopa* cf. *nudigena* and other flower visitors of *C. sandersonii* in South Africa (Table 1). The fraction of Milichiidae attracted to the other three mixtures was lower (52%–66%) and did not include pollinating species. In the complete mixture, the proportions of attracted fly taxa were comparable to mix A (88% Milichiidae); however, no pollinator of *C. sandersonii* was attracted. Differences between pollinating fly species and flies attracted to the complete mixture could be explained by a year effect, since the complete mixture was tested in 2013 but the majority of flower visitors were collected in 2014. A similar year effect could explain the observed differences in the species composition of milichiid flies attracted to the complete mixture and to the depleted mix A. The complete mixture was tested in 2013, whereas bioassays with depleted mixtures were performed in 2014.

With the exception of a few compounds such as (*E*)-2-octen-1-yl acetate that appear not to have been previously reported in floral scents, many of the compounds identified in *C. sandersonii* are widespread floral scent compounds (e.g., (*Z*)-3-hexen-1-yl acetate, hexyl acetate, benzyl acetate, linalool; compare [24, 25]) and insect pheromones and secretions [25, 44]. However, the qualitative composition is unusual for a flowering plant. For example, the biologically active compounds geraniol, 2-heptanone, and 2-nonanol are only presently known to be emitted in combination by some *Ophrys* orchids [45]. Moreover, we are not aware of any flowering plant other than *C. sandersonii* or any insect other than the honeybee that emits geraniol, 2-heptanone, 2-nonanol, and (*E*)-2-octen-1-yl acetate in combination. This observation further supports the hypothesis that *C. sandersonii* chemically mimics the volatile composition of honeybees under attack. Interestingly, isoamyl acetate, the main chemical component of defending honeybee volatiles [35], is not found in the floral scent. Furthermore, this compound was not EAD active to *Desmometopa* flies (Table S1). Thus, it seems that *Desmometopa* does not use this compound as a food source cue.

The mimicry of odor cues emitted by honeybees for pollinator attraction has also been reported in *Dendrobium sinense* Tang & F.T. Wang, a deceptive, non-rewarding orchid species [46]. This orchid mimics alarm pheromone components of the Asian honeybee *Apis cerana* Fab. and is pollinated by *Vespa bicolor* Fab., a hornet that hunts Asian honeybees as food for its larvae. For prey location, *V. bicolor* uses (*Z*)-11-eicosen-1-ol, a compound that is present not only in the sting gland [47] but also on the surface of the Asian honeybee [46]. Despite the similarity in mimicking *Apis* components, there are obvious differences between the two systems. *Ceropegia sandersonii* mimics the food source of adult carnivorous fly pollinators, whereas the orchid mimics the larval food of a wasp pollinator. Furthermore, *C. sandersonii* attracts several fly species, whereas the orchid attracts only a single wasp species. In accordance with the geographical distribution patterns of the deceptive plants and the bees, the orchid uses the Asian honeybee *A. cerana* as model, whereas *C. sandersonii* uses the western honeybee *A. mellifera*. In contrast to the hornet, which actively hunts foraging, non-alarming Asian honeybees, the kleptoparasitic flies depend on other predatory arthropods to get access to their food source. The highly volatile compounds released from honeybees caught by an arthropod signal a freshly killed prey item to the flies. As food stealers, there is a need for the flies to respond rapidly to the chemical cues that they use to locate their food

source before it is eaten by the predator. Indeed, a rapid response was observed in the bioassays, with flies often responding within seconds, consistent with this expectation.

Conclusions

In this study, we provide strong evidence for a new case of chemical mimicry whereby a plant species exploits the olfactory preference of scavenging, carnivorous *Desmometopa* flies. We show that the blend of volatiles emitted by *C. sandersonii* flowers is unique among flowering plants but similar to that released by attacked honeybees. Several of the compounds shared between flowers and bees were electrophysiologically active in antennae of fly pollinators. Furthermore, bioassays of a subset of these compounds elicited rapid attraction. The pollination system of *C. sandersonii* is functionally highly specialized because its floral scent is a chemical mimic of the western honeybee under attack, a food source for its kleptoparasitic fly pollinators.

EXPERIMENTAL PROCEDURES

Plant Material and Study Sites

Detailed information on plant material and study sites can be found in the Supplemental Experimental Procedures.

Flower Visitors and Pollinators

All methods used for observing and determining flower visitors and pollinators followed standard procedures and are described in detail in the Supplemental Experimental Procedures.

Volatile Collection and Chemical and Electrophysiological Analyses

Floral scent samples of *C. sandersonii* and samples of the potential chemical model (*Apis mellifera* L. under attack) were collected in situ with two different dynamic headspace methods. Thermal desorption (TD) samples were collected for identification of scent compounds, while solvent acetone (SAc) samples were collected for electrophysiological analyses. Floral scent samples were collected using standard approaches ([48]; Supplemental Experimental Procedures]. TD and SAc samples were analyzed with gas chromatographic and mass spectrometric (GC/MS) methods and/or electroantennographic approaches (GC/EAD) following standard protocols (see Supplemental Experimental Procedures).

Bioassays

To test whether kleptoparasitic pollinators are attracted to attacked honeybees, single foraging honeybees (Botanical Garden UBT, University of Bayreuth; three *A. m. carnica/ligustica* individuals in June 2011) were caught with an insect net. The bees were held within gauze and pressed between fingers, whereby they repeatedly extruded their stinger as a sign of defense when being attacked. As a control, individual bees were held within gauze but not pressed with fingers; these did not extrude their stinger.

Behavioral assays with electrophysiologically active geraniol, 2-heptanone, 2-nonanol, and (*E*)-2-octen-1-yl acetate were conducted to confirm whether they elicit behavioral responses. The synthetic substances were offered as a four-component (complete) mixture and tested against an acetone control. To further assess the importance of the single substances contained in the complete mixture, five-choice assays were performed with reduced mixtures, each of which omitted one substance (mix A, no (*E*)-2-octen-1-yl acetate; mix B, no 2-heptanone; mix C, no geraniol; mix D, no 2-nonanol). The four possible three-component mixtures were tested against each other and against the negative control (see Supplemental Experimental Procedures for details). An advantage of testing the mixtures simultaneously in choice experiments is that such tests clearly show preferences of flies without being influenced by factors (e.g., availability of flies) that might differ when testing the mixtures at different sites or times.

Statistical Analyses

All statistical analyses followed established methods. Detailed methods are outlined in the Supplemental Experimental Procedures.

SUPPLEMENTAL INFORMATION

Supplemental Information includes two figures, Supplemental Experimental Procedures, and one table and can be found with this article online at http://dx.doi.org/10.1016/j.cub.2016.07.085.

AUTHOR CONTRIBUTIONS

Conceptualization: A.H., S.D., and U.M.; Methodology: A.H., A.J., and S.D.; Investigation: A.H., A.J., I.B., M.v.T., S.D., S.D.J., and U.M.; Formal Analysis: A.H., S.D., and S.D.J.; Resources: M.G.; Writing – Original Draft: A.H.; Writing – Review & Editing: A.H., A.J., I.B., M.G., M.v.T., S.D., S.D.J., and U.M.

ACKNOWLEDGMENTS

The authors thank Irmgard Schäffler, Taina Witt, Adam Shuttleworth, David Styles, and Neil Crouch for assistance during field trips and three anonymous referees for helpful comments on earlier versions of the manuscript. This research was funded by a grant for PhD candidates according to the Bavarian Elite Aid Act (BayEFG). We dedicate this work to Prof. Dr. Stefan Vogel, who pioneered research on pollination of *Ceropegia*, and in remembrance of Hans-Jürgen Thorwarth, who dedicated a great part of his life to Asclepiadaceae and their illustration.

Received: April 5, 2016
Revised: July 1, 2016
Accepted: July 29, 2016
Published: October 6, 2016

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Current Biology, Volume *26*

Supplemental Information

Ceropegia sandersonii Mimics Attacked Honeybees to Attract Kleptoparasitic Flies for Pollination

Annemarie Heiduk, Irina Brake, Michael von Tschirnhaus, Matthias Göhl, Andreas Jürgens, Steven D. Johnson, Ulrich Meve, and Stefan Dötterl

Figure S1

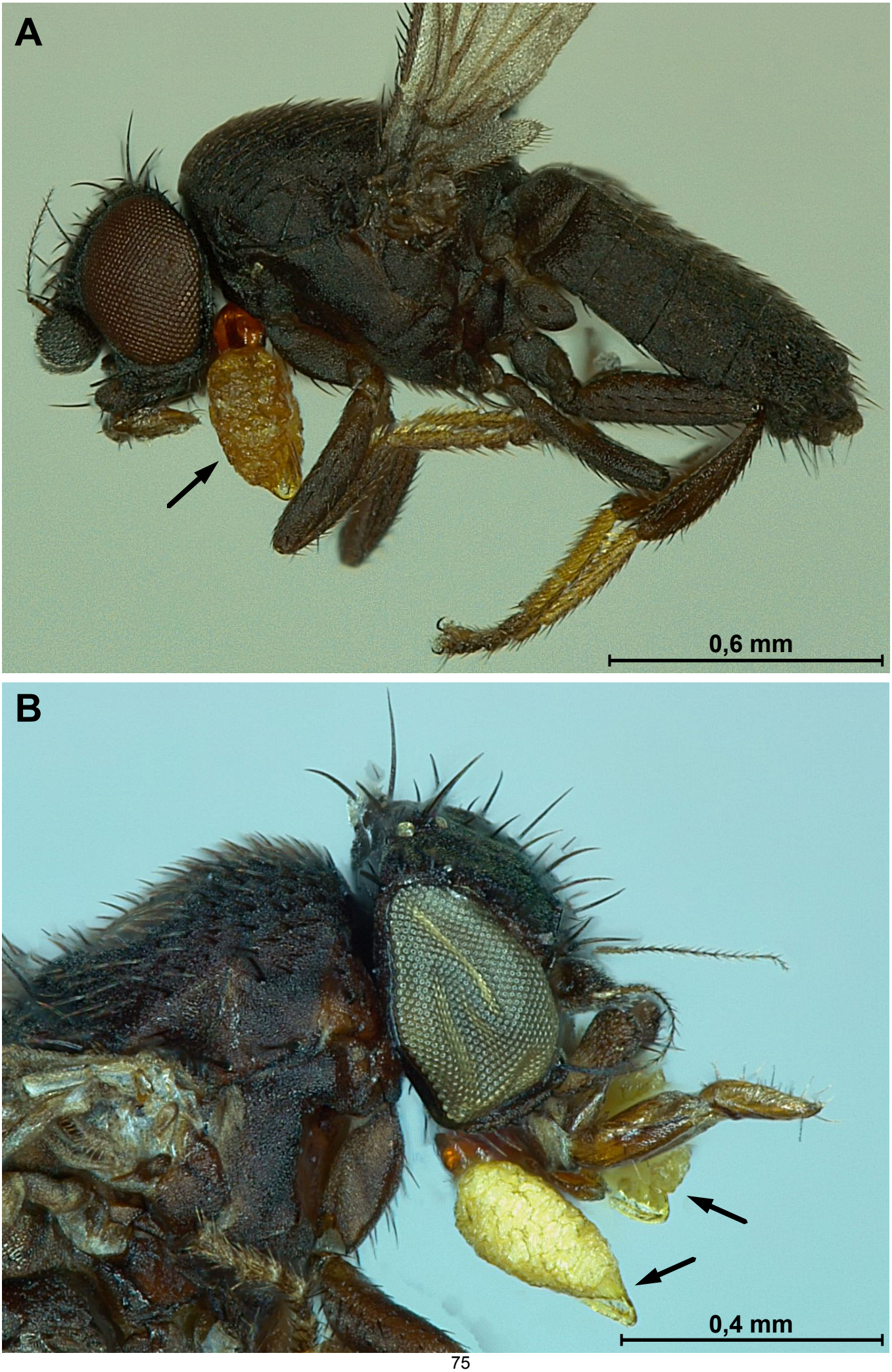
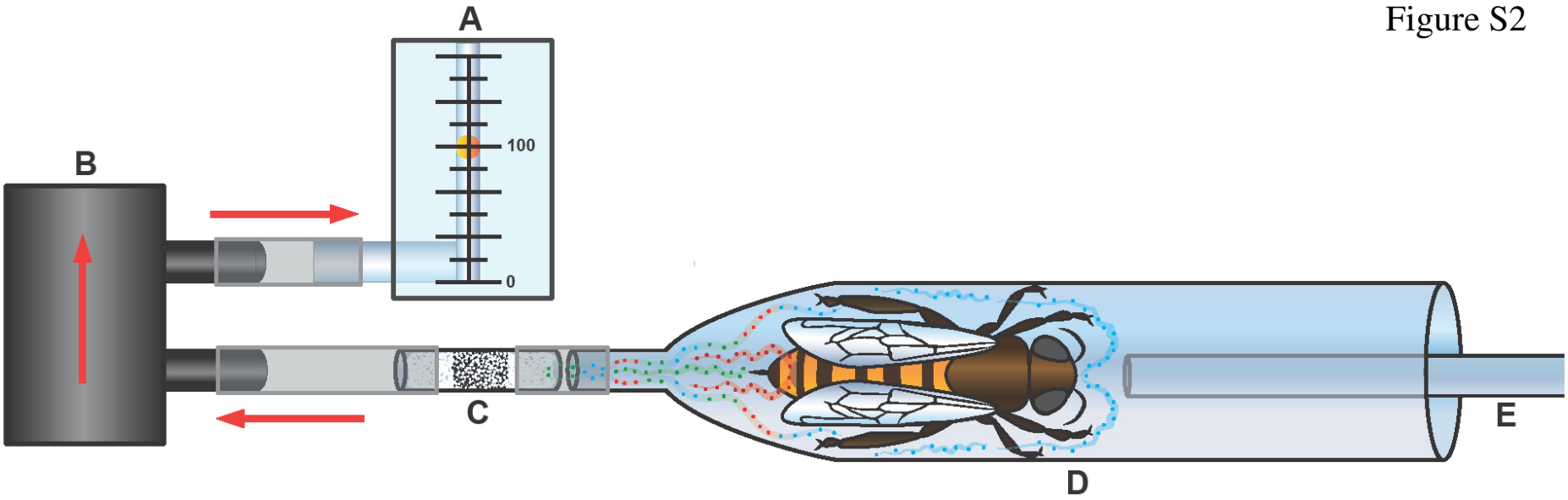


Figure S2



Supplemental Figure legends

Figure S1 (related to Table 1 and to Supplemental Experimental Procedures - *Flower visitors and pollinators*). *Desmometopa* cf. *nudigena* flies with pollinaria attached to their mouthparts.

Flies were collected from flowers of *Ceropegia sandersonii* in KwaZulu-Natal, South Africa. **A:** habitus. **B:** close up of a head. Arrows indicate pollinia of attached pollinaria.

Figure S2 (related to Figure 1, Table 2, and to Supplemental Experimental Procedures - *Volatile collection – Western honey bees*). Experimental setup for collection of volatiles from honey bees under simulated attack.

A: Flow meter, **B:** pump, **C:** adsorbent tube, **D:** glass tube with honey bee, **E:** glass pipette to simulate an attack. Flow meter, pump, adsorbent tube, and glass tube with honey bee are connected via silicone tubes. Red arrows indicate direction of air flow. Colored dots indicate volatile molecules released from sting (green), Nasonov (red), and mandible (blue) glands.

Supplemental Experimental Procedures

Plant material and study sites

The genus *Ceropegia* L. is restricted to the Old World and comprises more than 180 species [S11]. The plants grow in tropical and subtropical habitats from Canary Islands and West-Africa as far as Australia. The maximum diversity occurs in south-east Africa, India, Madagascar and China [S12]. *Ceropegia sandersonii* Decne. ex Hook. f. is widespread, typically found in dry scrub and forest or bushveld of South Africa (Eastern Cape, KwaZulu-Natal, Mpumalanga, Mozambique and Swaziland). For the present study plants from natural populations were investigated at four different localities in KwaZulu-Natal, South Africa (Muden: 28°58'23.8"S; 30°24'21.0"E; Inanda Dam: 29°40'25.6"S; 30°51'01.7"E; Ashburton: 29°39'23.75"S; 30°27'32.05"E; Kloof: 29°47'30.04"S; 30°50'04.88"E). In addition, we investigated *C. sandersonii* plants cultivated within the range of its natural distribution (Botanical Garden of the University of KwaZulu-Natal [BG-UKZN], Pietermaritzburg, South Africa), and plants grown in greenhouses outside the natural distribution range (Europe: University of Bayreuth [UBT], Germany; Botanical Garden of the University of Salzburg [BG-SBG], Austria).

Flower visitors and pollinators

Flower visitors and pollinators of *C. sandersonii* were observed from January to March 2013 and 2014 in its native range in South Africa and from May to October 2010-2012 and August/September 2014 in its non-native range at UBT and BG-SBG, respectively. Flies trapped inside flowers were collected and preserved in a 4% solution of glycerin in ethanol (99.8%) for later identification to family level. Milichiidae and Chloropidae were further identified to genus, morphospecies or species level. Collected flies were also examined for presence of

pollinaria on their body. A pollinarium is formed by two pollinia connected via caudicles and the corpusculum, and is clipped to the flies' mouthparts as a whole. During the pollination process flies insert a single pollinium of a pollinarium into a so called "guide rail" of the gynostegium, the synorganized reproductive organs [S13]. Flies carrying pollinaria (corpuscula with no, one, or two pollinia; cf. Figure S1) were treated as pollinators [S14].

Volatile collection

Flowers

To obtain thermal desorption (TD) samples of *Ceropegia sandersonii* flower scent, volatiles were collected from single newly opened flowers (BG-UKZN: three plant individuals, three flowers, one flower was sampled twice; UBT: two plant individuals, three flowers per plant, one sample each) as described by [S15]. Single flowers were enclosed in polyester oven bags (Toppits®, Germany) for a minimum of 10 min (UBT samples) and up to 20 min depending on the intensity of scent as perceived by the human nose. The accumulated floral volatiles were trapped by pulling air from the bag through small adsorbent tubes for 5 min using a membrane pump (G12/01 EB, Rietschle Thomas Inc., Puchheim, Germany) at a flow rate of 200 ml/min. The adsorbent tubes were made of ChromatoProbe quartz microvials of Varian Inc. (length: 15 mm, inner diameter: 2 mm), from which the closed end was cut off. These tubes were filled with a mixture of 1.5 mg Tenax-TA (mesh 60-80) and 1.5 mg Carbotrap B (mesh 20-40) (both Supelco, Bellefonte, PA, USA) embedded in glass wool. Additional samples of the surrounding air were collected to distinguish between floral scent compounds and compounds in the ambient air.

To obtain SAc-samples for electrophysiological analyses 18 individual *C. sandersonii* flowers from greenhouse plants in Bayreuth were enclosed into separate oven bags as described above. The emitted scent was trapped using larger adsorbent tubes (glass capillaries; length: 8 cm, inner diameter: 2.5 mm) filled with 15 mg Tenax-TA (mesh 60-80) and 15 mg Carbotrap B (mesh 20-40). The scent was trapped for 6 hrs during daytime (9 am to 6 pm) at a flow rate of 100 ml/min. The volatiles trapped on an adsorbent tube were eluted with 60 µl of acetone (SupraSolv, Merck KgaA, Germany). Samples were pooled to finally obtain three SAc-samples (SAc1: one flower in ~50 µl; SAc2: six flowers in ~300 µl; SAc3: eleven flowers in ~550 µl) for electrophysiological measurements.

Western honey bees

To obtain TD-samples of honey bees under attack, foraging worker bees (*Apis mellifera*) were caught at the BG-SBG in summer 2015 (23 individuals, *A. m. carnica/ligustica*) and at the BG-UKZN in May 2016 (five individuals, *A. m. scutellata;* this subspecies co-occurs *with C. sandersonii*). Single bees were inserted, abdomen first, in glass tubes (inner diameter: 5.4 mm) and "attacked" with the tip of a glass pipette (Figure S2). As consequence, the bees a) bit into the glass pipette, and b) extruded their sting and tried to pierce the glass tube. The volatile compounds released from these bees were collected for two minutes per bee in an adsorbent trap (BG-SBG, 2015: one sample collected from five bees; three samples collected from six bees each, flow rate 200ml/min; BG-UKZN, 2016: five samples collected from a single bee each, flower rate 50ml/min) which was coupled to the glass tube at the side of the bee's abdomen. To specifically identify alarm components, control samples were taken from each *A. m. carnica/ligustica* individual before being "attacked" with a glass pipette. Samples of *A. m. scutellata* were analyszd against an ambient control sample. To obtain a SAc-sample of honey

bees, volatiles were collected in a similar way as described for collecting TD-samples. However, only the abdomen was inserted in a glass tube and bees were hold between fingers in order to simulate a predator attack. Volatiles were collected into a larger adsorbent tube. In total, volatiles of 12 bees (*A. m. carnica/ligustica*, caught at UBT in summer 2011) were trapped in one adsorbent tube and eluted with 70 µl of acetone (SupraSolv, Merck KgaA, Germany) resulting in a sample of ~60 µl available for electrophysiological measurements.

Chemical analyses

TD-samples collected at UBT were analyzed by gas chromatography / mass spectrometry (GC/MS) on a Varian Saturn 3800 gas chromatograph (GC) fitted with a 1079 injector and a ZB-5 column (5 % phenyl polysiloxane, length: 60 m, inner diameter: 0.25 mm, film thickness: 0.25 µm, Phenomenex), and a Varian Saturn 2000 mass spectrometer (MS). The adsorbent tubes were inserted via Varian's Chromatoprobe into the GC injector [S15, S16]. The injector split vent was opened and the injector was heated to 40°C to flush any air from the system. After 2 min the split vent was closed and the injector heated at 200°C/min to 200°C, then held at 200°C for 1.7 min, after which the split vent was opened and the injector heated to 250°C (to condition the adsorbent tubes for further scent collections) until the end of the run. Electronic flow control was used to maintain a constant helium carrier gas flow rate (1.0 ml/min). The GC oven temperature was held for 4.5 min at 40°C, then increased by 6°C/min to 260°C and held for 3 min at this temperature. The mass spectra were taken at 70 eV with a scanning speed of 1 scan/s from m/z 30 to 350. SAc-samples collected at UBT were analyzed with the same GC/MS setup and settings as described above for TD-samples. 1.0 µl of each SAc-sample was syringed into a small adsorbent tube (see above), which was placed in the injector port by means of the ChromatoProbe. Processing of the data was performed by the Saturn Software package 5.2.1.

TD-samples collected in South Africa were analyzed using a Bruker 450 GC (Varian, Palo Alto, California) with a 30 m x 0.25 mm internal diameter (film thickness: 0.25 µm) Bruker DB5 column connected to a 11 m Bruker DB1 column (film thickness: 0.25 µm) coupled to a Bruker 350 quadrupole mass spectrometer in electron ionization mode at 70 eV. TD-samples were placed in a Varian 1079 injector equipped with a Chromatoprobe thermal desorption device [S17]. The flow of helium carrier gas was 1.6 ml/min. The injector was held at 50°C for 2 min with a 20:1 split and then increased to 200°C at 200°C/min in splitless mode for thermal desorption of samples. After a 3 min hold at 50°C, the temperature of the GC oven was ramped up to 240°C at 10°C/min and held at this temperature for 12 min.

TD-samples of honey bees under simulated attack collected in Salzburg were analyzed using an automatic TD-20 system (Shimadzu, Japan) coupled to a Shimadzu GC/MS-QP2010 Ultra equipped with a ZB-5 fused silica column (5% phenyl polysiloxane; length: 60 m, inner diameter: 0.25 mm, film thickness: 0.25 µm, Phenomenex). The samples were run with a 1:1 split and a constant helium carrier gas flow of 1.5 ml/min. The GC oven temperature started at 40°C, then increased by 6°C/min to 250°C and held for 1 min. The MS interface worked at 250°C. Mass spectra were taken at 70 eV (EI mode) from m/z 30 to 350. GC/MS data were processed using the GCMSolution package, Version 2.72 (Shimadzu Corporation 2012).

Solvent acetone (SAc) samples of honey bees under simulated attack were analyzed by GC/MS using a Shimadzu GCMS-QP2010 Ultra equipped with an AOC-20i auto injector (Shimadzu, Tokyo, Japan) and again a ZB-5 fused silica column (5% phenyl polysiloxane; length: 30 m, inner diameter: 0.32 mm, film

thickness: 0.25 µm, Phenomenex). 1.0 µl of the samples was injected (injection temperature: 220°C; split ratio 1:1), and the column flow (carrier gas: helium) was set at 3 ml/min. The GC oven temperature was held at 40°C for 1 min, then increased by 10°C/min to 220°C and held for 2 min. The MS interface worked at 220°C. Mass spectra were again taken at 70 eV (in EI mode) from m/z 30 to 350 and data processed as described above.

Scent components of the GC/MS spectra were identified using the mass spectral data bases NIST 11, Wiley 9, MassFinder 3, FFNSC 2, and Adams [S18]. Where possible, compounds were verified using retention times and mass spectra of authentic standards (purchased from Sigma Aldrich) or by comparison with published Kovats retention indices (KRI). For estimation of total scent emission, known amounts of monoterpenoids, aromatics, and aliphatics (applied to small adsorbent tubes) were injected, and the mean peak area of these compounds was used for quantification [S15].

Electrophysiological analyses (GC/EAD)

The flower scent of *C. sandersonii* was tested on the antennae of nine *Desmometopa sordida* flies (SAc3: eight runs with five females and two males; antenna of one male was used twice; SAc1: two runs with two female flies) collected from *C. sandersonii* flowers (UBT, 2010 and 2011), and 19 *D. microps* flies (SAc2: 25 runs with 13 females and six males; antennae of two females and two males were used twice, of one male and one female both antennae were used) collected from inflorescences of *Solidago canadensis* (BG-SBG, 2013). The SAc-sample of honey bees under simulated attack was tested on the antennae of three female *D. sordida* flies (one run per antenna) collected from *C. sandersonii* flowers (UBT, 2011). Some of the measurements did not deliver clear results and were excluded from the analyses. The number of fly individuals and antennae finally analyzed is given in Table 2 and Table S1.

The analyses with *D. sordida* and *D. microps* flies were performed with a Carlo Erba Vega 6000 Series 2 (Rodano, Italy) and an Agilent 7890A (Santa Clara, California, USA) gas chromatograph, respectively. Both GCs were equipped with a flame ionization detector (FID) and an EAD setup (heated transfer line, 2-channel USB acquisition controller) provided by Syntech (Kirchzarten, Germany). For each run an acetone scent sample (flower scent: 1.0 µl, alarm pheromone: 1.5 µl) was injected in splitless mode (injector temperature: 250°C; oven temperature: 40°C). In both systems, the split opened 30 sec after injection, and the oven heated by 10°C/min to 220°C. Both GCs were equipped with a Zebron ZB-5 column for analysis (5% phenyl polysiloxane; length: 30 m, inner diameter: 0.32 mm, film thickness: 0.25 µm, Phenomenex). In the Carlo Erba GC, the column was split at the end by a four-arm flow splitter (GRAPHPACK 3D/2, Gerstel, Mühlheim, Germany) into two deactivated capillaries (length: 50 cm, inner diameter: 0.32 mm) leading to the FID and to the EAD setup. Helium was introduced as a make-up gas through the fourth-arm splitter. In the Agilent GC, the column was split at the end by a µFlow splitter (Gerstel, Mühlheim, Germany; nitrogen was used as make-up gas) into two deactivated capillaries, one (length: 2 m, inner diameter: 0.15 mm) again leading to the FID setup, the other (length: 1 m, inner diameter: 0.2 mm) leading to the EAD setup. In both GC systems the outlet of the EAD was placed in a cleaned, humidified air flow directed over the fly antenna.

Flies used for measurements were anaesthetized (CO_2) and their heads cut off. Two glass micropipette electrodes were filled with insect Ringer's solution (8.0 g/l NaCl, 0.4 g/l KCl, 4.0 g/l $CaCl_2$) and connected to silver wires.

The caudal side of the head was connected to the reference electrode, and the recording electrode was placed in contact with the antenna tip (first flagellomere).

To identify the EAD-active compounds, 1.0 µl each of the SAc-samples was analyzed by GC/MS (see above).

Bioassays

Geraniol (Sigma-Aldrich, 98%), 2-heptanone (Merck, >98%), and 2-nonanol (Sigma-Aldrich, 99%) were commercially available, and (*E*)-2-octen-1-yl acetate was synthesized by acetylation with acetic anhydride and pyridine in CH_2Cl_2 under DMAP catalysis ([S19]; purity >99%). The synthetic substances were offered as a four component mixture (henceforth complete mixture; equal volumes, dilution in acetone: 10^{-2}; v/v). The complete mixture was offered in a 1 ml glass vial (Supelco) partially pushed into soil and tested against a similarly placed control glass vial containing an equal amount of acetone only. The two vials were placed 30 cm apart and each assay lasted a minimum of 30 min and up to 60 min. The position of test sample and control was exchanged at half-time. As determined by dynamic headspace and GC/MS, the average amount of scent released from the test vial containing the complete mixture was a good approximation of the amount of scent released from 1-3 flowers. Approaching flies were caught with a small insect net when flying or sitting within a radius of 10 cm around the vials. This bioassay was conducted nine times on nine different days and at two different sites in South Africa (BG-UKZN: n = 6; Muden: n = 3) in February/March 2013.

Five-choice assays with reduced mixtures (Mix A: no (*E*)-2-octen-1-yl acetate, Mix B: no 2-heptanone, Mix C: no geraniol, and Mix D: no 2-nonanol) were performed eight times on eight different days and two different sites in South Africa (BG UKZN: n = 6; Muden: n = 2) in February/March 2014. The mixtures A-D and an acetone control were offered on sticky traps. A trap consisted of a black colored styrofoam square (9 cm x 9 cm) with a hole (8 mm diameter) in the center. The upper side of the square was covered with colorless insect glue (Tanglefoot®). A 1 ml glass vial (Supelco) was filled with 700 µl of the sample (dilution: 10^{-2}; v/v) and inserted with its' neck into the hole from below. A wooden stick (25 cm length) was taped to the sample vial to place the trap in ~20 cm height above ground. In each assay the traps were placed with a distance of 6 m to each other and offered for at least 4 hours and up to 8 hours. Flies were collected from the sticky traps, rinsed with kerosene to remove the insect glue, and stored and identified as described before.

Statistical analyses

Bioassay data were analyzed using generalized linear models implemented in SPSS 21 (IBM Corp.). Differences in the mean counts of insects attracted to each volatile mixture were analyzed in models with a negative binomial distribution and log link function. For experiments that involved several trials, each trial was treated as the subject in generalized estimating equations (GEE) to account for lack of independence in responses during each trial. These models used an exchangeable correlation matrix to account for correlations within trials and Wald statistics to assess significance. For graphical representation of marginal (adjusted) means and standard errors, we used back-transformed values, resulting in asymmetric standard errors. Post-hoc comparisons of the mean number of insects attracted to each mixture were obtained using the sequential Sidak method which is known to achieve a good balance between the possibilities of type 1 versus type 2 errors.

Differences in the overall frequencies of different fly taxa (Milichiidae, Chloropidae and other Diptera) attracted to the different mixtures were assessed using Fisher's exact test (SPSS 21). The frequencies were converted to percentages for graphical presentation. Control samples were excluded from statistical analysis as no flies responded, apart from a single fly in one assay.

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Publication 3: Floral scent and pollinators of *Ceropegia* trap flowers

Published 2017 in *Flora* (invited contribution)

Flora 232 (2017) 169–182

Contents lists available at ScienceDirect

Flora

journal homepage: www.elsevier.com/locate/flora

Floral scent and pollinators of *Ceropegia* trap flowers☆

Annemarie Heiduk[a,j], Irina Brake[b], Michael v. Tschirnhaus[c], Jean-Paul Haenni[d], Raymond Miller[e], John Hash[f], Samuel Prieto-Benítez[g], Andreas Jürgens[e,h], Steven D. Johnson[e], Stefan Schulz[i], Sigrid Liede-Schumann[j], Ulrich Meve[j], Stefan Dötterl[a,*]

[a] *Department of Ecology and Evolution, Plant Ecology, University of Salzburg, Salzburg, Austria*
[b] *Department of Life Sciences, Natural History Museum, London, United Kingdom*
[c] *Department of Biology, University of Bielefeld, Bielefeld, Germany*
[d] *Entomology, University of Neuchâtel, Neuchâtel, Switzerland*
[e] *School of Life Sciences, University of KwaZulu-Natal, Pietermaritzburg, South Africa*
[f] *Department of Entomology, University of California, Riverside, California 92521, USA*
[g] *Departamento de Biología y Geología, Física y Química Inorgánica, Universidad Rey Juan Carlos-ESCET, C/Tulipán, s/n., 28933 Móstoles, Madrid, Spain*
[h] *Department of Biology, Plant Chemical Ecology, Technische Universität Darmstadt, Darmstadt, Germany*
[i] *Institute of Organic Chemistry, Technische Universität Braunschweig, Braunschweig, Germany*
[j] *Department of Plant Systematics, University of Bayreuth, Bayreuth, Germany*

ARTICLE INFO

Article history:
Received 24 May 2016
Received in revised form
15 December 2016
Accepted 1 February 2017
Edited by Prof. AA Cocucci
Available online 4 February 2017

Keywords:
Fly pollination
Pollen transfer efficiency
Chemical mimicry
Scent specificity
Deceptive strategy

ABSTRACT

Ceropegia L. (Apocynaceae, Asclepiadoideae) comprises more than 200 species, all characterized by complex pitfall flowers. The deceptive flowers are myiophilous and pollinated predominantly by small flies from different families. It has been suggested that floral scent cues, that mimic food sources or oviposition sites, play an important role for attraction of target fly pollinators, and, together with morphological flower traits, explain the high functional specialization in terms of pollination by specific taxa. However, apart from two *Ceropegia* species, the floral scent composition and the mimicry strategies in this genus are unexplored. We tested for associations between floral scent and insect visitor and pollinator assemblages of 14 *Ceropegia* species. We also used nrDNA and chloroplast DNA markers to calculate a Maximum Likelihood tree and test for phylogenetic signal in scent chemistry and flower visitors/pollinators. The observed pollinators belonged to eight fly families, at least 18 genera, and 33 morphospecies, but each *Ceropegia* species was typically associated with only one or two pollinating fly families or genera. We detected a total of 317 floral volatiles, including aliphatic and aromatic components, terpenes, and various unknowns. Both flower visitor and pollinator patterns did not show an overall association with floral scent chemistry. There was phylogenetic signal in flower visiting fly families and fly pollinator assemblages, but not in flower visiting fly morphospecies and overall scent chemistry. We discuss that despite the not existing correlation between pollinator and scent patterns the highly specific pollination system in *Ceropegia* will be explained mainly by floral scent chemistry.

1. Introduction

Among plants that deceive their pollinators through chemical mimicry, the most fascinating ones are those with pitfall flowers to temporarily trap pollinators. The complex trap flowers of species in the genus *Ceropegia* L. (Apocynaceae, Asclepiadoideae) have aroused the interest of naturalists ever since they were discovered, and first records on flies as pollinators date back more than a century (Delpino, 1869; Knuth, 1909). Pioneer studies on flower structure, functional flower parts, and pollination biology were contributed by Müller (1926) and Vogel (1954, 1960, 1961). Vogel (1961) provided very detailed descriptions of the various specialized flower parts and tissues, and their functional interaction to achieve pollination by small flies, such as Ceratopogonidae, Chloropidae, Drosophilidae, Milichiidae, Phoridae,

☆ This article is part of a special feature entitled: "Patterns and mechanisms in plant-pollinator interactions" published at FLORA volume 232, 2017.

* Corresponding author at: Department of Ecology and Evolution, Plant Ecology, University of Salzburg, Hellbrunnerstr. 34, 5020 Salzburg, Austria.
E-mail address: stefan.doetterl@sbg.ac.at (S. Dötterl).

http://dx.doi.org/10.1016/j.flora.2017.02.001
0367-2530/

Fig. 1. Flowers of investigated *Ceropegia* species. **A:** *Ceropegia rupicola;* **B:** *C. stenantha;* **C:** *C. ampliata;* **D:** *C. denticulata;* **E:** *C. barklyi;* **F:** *C. carnosa;* **G:** *C. haygarthii;* **H:** *C. woodii;* **I:** *C. crassifolia;* **J:** *C. nilotica;* **K:** *C. pachystelma;* **L:** *C. cycniflora.* Photographs: U. Meve and A. Heiduk.

Scatopsidae, Sciaridae, and Tachinidae (e.g., Coombs et al., 2011; Heiduk et al., 2010, 2015; Ollerton et al., 2009; Vogel, 1961).

Despite the great diversity of sizes, shapes, ornamentation, colors and color patterns of *Ceropegia* flowers (Fig. 1), the basic flower structure is the same in all species (see Vogel, 1961) with extremely synorganized flower parts (Endress, 2015). The corona is fused to form a sophisticated pitfall flower with special radial symmetry, i.e., "revolver flower", where always five orifices provide entrance for flower visitors (Endress, 1994, 2015). The fused corolla can be divided into three functional parts: (1) the flower tip, where insects enter and which is often decorated with versatile hairs likely to increase visual attractiveness, (2) the slippery, hairy tube through which insects drop down and are hindered in their attempts to escape upwards again, and (3) the basal inflation that contains the fused male and female reproductive organs (i.e., gynostegium), and which imprisons the visitors for several hours (see Vogel, 1961).

In general, each *Ceropegia* species has a functional group of fly pollinators belonging to only one or few genera and/or families (Heiduk et al., 2015; Ollerton et al., 2009). This specialization seems to be mainly achieved by floral scent, though also morphological filters occur (Masinde, 2004). If perceivable by the human nose, the description of *Ceropegia* flower scents ranges from pleasantly sweet and fruity, via soapy and leather-like, to pungent acidic and disgustingly putrid. Vogel (1961) demonstrated that specialized epithelia (i.e., 'osmophores') on the corolla tips are responsible for the emission of flower scent, and this was later confirmed by Heiduk et al. (2010). *Ceropegia* plants often grow hidden in bushes, and thus Vogel (1961) suggested that floral scent has to play a major role in attracting the fly pollinators from a distance. As flowers of *Ceropegia*

neither offer nectar in detectable quantities nor pollen grains that could be insalivated, flies are attracted to the flowers by deceit and it was suggested that floral scent in *Ceropegia* is a chemical mimic of (decaying) plant, animal, or fungus material (but see Coombs et al., 2011; Vogel, 1961, 1993). Despite of its likely importance as the main mediator, floral scent has not been well described in *Ceropegia*. Specific attractive components have only been identified for Chinese *C. dolichophylla* (Heiduk et al., 2010, 2015), and South African *C. sandersonii* (Heiduk et al., 2016). Both species are pollinated by kleptoparasitic *Desmometopa* flies (Milichiidae) and have a kleptomyiophilous mimicry strategy, i.e., they deceive kleptoparasitic flies through chemical mimicry of a food source of adults. In *C. sandersonii* the model mimicked is a "honey bee under attack" (Heiduk et al., 2016), but for *C. dolichophylla* the model could not be defined precisely. For all other *Ceropegia* species the floral scents, mimicry strategies, and models mimicked remain unknown.

The objectives of our study on *Ceropegia* species were (1) to identify the flower visiting and pollinating flies, and (2) to analyze the floral scent composition using dynamic headspace and gas chromatography coupled to mass spectrometry (GC/MS). We also (3) looked for relationships between floral scent and visitor/pollinator patterns, and (4) checked for phylogenetic signals by comparing scent and pollinator data of all species with a computed phylogeny of the plants. (5) For seven *Ceropegia* species the reproductive success was characterized by calculating the Pollen Transfer Efficiency (PTE).

2. Methods and material

2.1. Plant material and study sites

Ceropegia includes more than 200 described species restricted to tropical and subtropical habitats of the Old World. The centers of diversity are southeast Africa, India, Madagascar and China (e.g., Meve and Liede-Schumann, 2007; Punekar et al., 2013). We included 14 species in this study: *C. ampliata* E. Mey., *C. barklyi* Hook. f., *C. carnosa* E. Mey (incl. variants formerly regarded as distinct species, *C. racemosa* N. E. Br.), *C. crassifolia* Schltr., *C. cycniflora* R. A. Dyer, *C. denticulata* K. Schum. ex Engl., *C. dolichophylla* Schltr., *C. haygarthii* Schltr., *C. nilotica* Kotschy, *C. pachystelma* Schltr., *C. rupicola* Deflers, *C. sandersonii* Decne. ex Hook. f., *C. stenantha* K. Schum., and *C. woodii* Schltr. (Fig. 1). Plants either grew in natural populations or were cultivated within or outside their natural distribution range. Scent and pollinator data for *C. dolichophylla* (Heiduk et al., 2015) and *C. sandersonii* (Heiduk et al., 2016) were obtained from previous studies.

Plants of *C. barklyi*, *C. carnosa*, *C. crassifolia*, *C. cycniflora*, *C. haygarthii*, *C. nilotica*, *C. pachystelma*, and *C. woodii* were investigated from January till March 2013 and 2014 within their range of natural distribution in KwaZulu-Natal, South Africa (Ashburton: 29°39′23.75″S; 30°27′32.05″E; Inanda Dam: 29°40′25.6″S; 30°51′01.7″E; Kloof: 29°47′30.04″S; 30°50′04.88″E; Muden: 28°58′S; 30°24′E; Pietermaritzburg: Botanical Garden of the University of KwaZulu-Natal [BG-UKZN]). Flowers of *C. stenantha* were collected in Rukwa, Tanzania (6°51′03.94″S; 31°11′41.57″W) in December 2012 and January 2013 for analyzing their trap catches. For *C. carnosa* additional flowers were collected in Dano, Bukina Faso (11°02′51.5″N 3°05′46.3″W) in September 2013, again to analyze their trap catches.

Plants of *C. ampliata*, *C. denticulata*, *C. rupicola*, and *C. stenantha* were cultivated in greenhouses outside their natural distribution range (Germany: University of Bayreuth [UBT], summer 2010–2012; Austria: Botanical Garden of the University of Salzburg [BG-SBG], summer 2013) and used for pollinator observations and/or scent sampling. For *C. ampliata* additional flowers were collected in summer 2009 from a plant growing in a private collection in Blaubeuren, Germany, for analyzing their trap catches.

2.2. Flower visitors, pollinators and pollen transfer efficiency (PTE)

To obtain information on visitors and pollinators of studied *Ceropegia* species, flowers were collected during the day and stored in ethanol for later investigation.

Insects inside the flowers were removed and examined for pollinaria (corpuscula with no, one, or two pollinia) clipped to their bodies. Only insects that carried pollinaria were denoted as pollinators (see Ollerton et al., 2009). All insects were stored in a 4% solution of glycerin in ethanol (99.8%), and Diptera were later identified to family, genus, and/or morphospecies (includes identified species) level.

For seven *Ceropegia* species, i.e. *C. barklyi* (n = 5), *C. crassifolia* (n = 2), *C. haygarthii* (n = 1), *C. nilotica* (n = 1), *C. pachystelma* (n = 5), *C. carnosa* (n = 7), and *C. woodii* (n = 1), pollination success was investigated in natural habitats (KwaZulu-Natal). Flowers were picked from plants in the afternoon/evenings (between January and March 2013 and 2014) and gynostegia were checked for pollinaria removal and pollinia insertion. For each species the mean number of inserted pollinia and the mean number of removed pollinaria were calculated. Reproductive success was determined as the percentage of removed pollinia that were inserted between guide rails (compare Johnson et al., 2005). The mean number of inserted pollinia was divided by twice the mean number of removed pollinaria, because each pollinarium consists of two pollinia (for application in *Ceropegia* see Coombs et al., 2011; Heiduk et al., 2015). Pollen transfer efficiency (PTE) was calculated as

$$PTE = p_i/(2 \times P_r),$$

where p_i is the mean number of inserted pollinia, and P_r is the mean number of removed pollinaria. In each species, data of 2013 and 2014 were pooled, and different locations were combined to the habitat "KwaZulu-Natal".

2.3. Collection of floral volatiles

To obtain headspace samples for thermodesorption (TD), floral volatiles were collected from newly opened flowers as described by Dötterl et al. (2005). Single flowers (or in some cases a group of flowers) were enclosed in polyester oven bags (Toppits®, Germany) for a minimum of 5 min and up to 110 min, depending on the intensity of scent as perceived by the human nose. The accumulated floral volatiles were trapped by pulling air from the bag through small adsorbent tubes for 2 min and up to 30 min using a membrane pump (G12/01 EB, Rietschle Thomas Inc., Puchheim, Germany) at a flow rate of 200 ml/min. The adsorbent tubes were made of ChromatoProbe quartz microvials of Varian Inc. (length: 15 mm, inner diameter: 2 mm), from which the closed end was cut off. These tubes were filled with a mixture of 1.5 mg Tenax-TA (mesh 60–80) and 1.5 mg Carbotrap B (mesh 20–40) (both Supelco, Bellefonte, PA, USA) embedded in glass wool. Additional samples of the surrounding air were collected to distinguish between floral volatiles and volatiles in the ambient air (compare with Heiduk et al., 2015).

2.4. Chemical analyses

Samples were analyzed over several years and in different labs with different equipment. Previous studies have shown that analytical equipment has little influence on the results of analysis of scent blends (Johnson, unpublished data). TD-samples collected in South Africa in 2013 were analyzed by GC/MS using a Bruker 450 GC (Var-

ian, Palo Alto, California) with a Bruker DB5 column (length: 30 m, inner diameter: 0.25 mm, film thickness: 0.25 μm) connected to a Bruker DB1 column (length: 11 m, film thickness: 0.25 μm) coupled to a Bruker 350 quadrupole MS.

TD-samples collected in South Africa in 2014 were analyzed on a Bruker 450 GC with an Alltech Carbowax column (length: 30 m, inner diameter: 0.25 mm, film thickness: 0.25 μm), connected to a 11 m Bruker DB1 column (inner diameter: 0.25 mm, film thickness: 0.25 μm) coupled to a Bruker 300 quadrupole MS in electron-impact ionization mode at 70 eV. TD-samples were placed in a Varian 1079 injector equipped with a Chromatoprobe thermal desorption device (Amirav and Dagan, 1997). The flow of helium carrier gas was 1.0 ml/min. The injector was held at an initial temperature of 40 °C for 2 min with a 20:1 split and then increased to 200 °C at 200 °C/min in splitless mode for thermal desorption. After a 3 min hold at 40 °C, the temperature of the GC oven was ramped up to 240 °C at 10 °C/min and held for 12 min. Processing of data was performed using Varian Workstation Software.

TD-samples collected in Bayreuth were analyzed using a Varian Saturn 3800 GC fitted with a 1079 injector (ZB-5 column, 5% phenyl polysiloxane, length: 60 m, inner diameter: 0.25 mm, film thickness: 0.25 μm, Phenomenex) and a Varian Saturn 2000 MS (for further details see Heiduk et al., 2010). Processing of the data was performed by the Saturn Software package 5.2.1.

TD-samples collected in Salzburg were analyzed using a Shimadzu GC/MS-QP2010 Ultra (ZB-5 fused silica column, 5% phenyl polysiloxane; length: 60 m, inner diameter: 0.25 mm, film thickness: 0.25 μm, Phenomenex) equipped with an automatic TD system (TD-20, Shimadzu, Japan) (for details see Heiduk et al. (2015) and Mitchell et al. (2015)). GC/MS data were processed using the GCMSolution package, Version 2.72 (Shimadzu Corporation, 2012).

Identification of scent components was carried out using the mass spectral data bases NIST 11, Wiley 9, MassFinder 3, FFNSC 2, and Adams (2007). Whenever possible, components were verified using retention times and mass spectra of authentic standards or by comparison with published Kovats retention indices (KRI). 3-Acetyloxy-4-phenylbutan-2-one and 3-acetyloxy-1-phenylbutan-2-one, 1-phenyl-2,3-butandione, and 3-hydroxy-1-phenylbutan-2-one were available in the standard collection of SS. For estimation of total scent emission, known amounts of monoterpenoids, aromatic and aliphatic components (applied to small adsorbent tubes) were injected, and the mean peak area of these components was used for quantification (Dötterl et al., 2005).

2.5. Genetic analyses

The Internal Transcribed Spacer (ITS) of nrDNA and five chloroplast DNA markers (*trn*T-L, *trn*L-F and *trn*H-*psb*A spacers and the *trn*L and *rps*16 intron) were obtained from all 14 *Ceropegia* species and mostly from the accessions for which scent was studied (Table S1) following the procedures detailed in Meve and Liede-Schumann (2007). For *C. ampliata*, *trn*T-L spacer and for *C. woodii*, *rps*16 intron sequences could not be obtained. Sequences were aligned with the OPAL package (Wheeler and Kececioglu, 2007) of Mesquite 3.0 (Maddison and Maddison, 2015) and concatenated. Sequence characteristics are provided in Table S2. The Maximum Likelihood (ML) tree was calculated on the CIPRES Platform (Miller et al., 2010) using RAxML-HPC v. 8.2.9 (Stamatakis, 2014) on BlackBox using a mixed partition model for the six data partitions, and standard settings.

2.6. Statistical analyses

For each floral scent sample the relative amount contributed (percentage of the total peak area) was determined for each component and used for further analyses. If more than one sample was taken from the same individual flower or plant individual, the mean relative amounts per component were calculated and used for analyses.

To test for semi-quantitative differences in floral scent profiles among the 14 *Ceropegia* species, the Bray-Curtis (BC) similarity index was calculated using Primer 6.1.11 (Clarke and Gorley, 2006). Based on the BC-matrix, an ANOSIM (Factor: *Species*; 10,000 permutations) was performed using the same software package to test for differences in scent among species. Nonmetric multidimensional scaling (NMDS) was used in Primer for graphical display of variation in floral scent among plant individuals (Clarke and Gorley, 2006).

To test whether phylogenetic relatedness influenced floral scent (Prieto-Benítez et al., 2016) and flower visiting/pollinating flies, we estimated phylogenetic signal using K_{mult} (Blomberg et al., 2003), a generalized variant of multivariate Blomberg's K (Blomberg et al., 2003; K_{mult}; Adams, 2014). Phylogenetic signal was tested in the multivariate dataset of floral scents (mean relative amount of components), and the multivariate datasets of flower visiting/pollinating fly taxa (family and morphospecies level). The expected evolution of traits undergoing Brownian motion model is indicated by K_{mult} of ~1. Significance of phylogenetic signal was determined by comparing observed with expected phylogenetic signal (running 999 permutations).

To test for linkage between floral scent and fly taxa, we correlated a BC similarity matrix based on floral scent (species means in relative amounts per compound were used for calculation) to BC similarity matrices of fly visitors and fly pollinators (family and morphospecies level) using RELATE in Primer (Spearman's rank correlation, 10,000 permutations). In cases where fly visitors/pollinators from both the native and the non-native range were available, only data from native range were used for calculations.

3. Results

3.1. Flower visitors, pollinators and pollen transfer efficiency PTE

The flower visitors/pollinators of the 14 *Ceropegia* species were exclusively Diptera from at least 40 morphospecies of ten different families (Anthomyiidae, Ceratopogonidae, Chloropidae, Drosophilidae, Lauxaniidae, Milichiidae, Muscidae, Phoridae, Scatopsidae, and Tachinidae; Table 1). Flowers had a mean of four flies trapped (based on flowers with positive trap catches) and a maximum of 54 individuals. Except for flowers of *C. ampliata*, which contained larger flies (>3 mm) of the families Muscidae, Tachinidae and Anthomyiidae (Table 1), the flower visitors were mostly less than 3 mm in length. In seven of the 14 species, flies of a single family were found trapped in flowers, and seven species contained flies from two or more families (Table 1). Generally, flower visitors, at the morphospecies level, differed among plant species, whereas there was an overlap at genus and family level among some of the *Ceropegia* species.

Pollinaria were found on 33 of the fly morphospecies, exclusively on the mouthparts, and these flies are considered as pollinators (Table 1). For nine of the studied species these pollinator flies belonged to a single family (e.g., Ceratopogonidae in *C. barklyi*, *C. pachystelma*, and *C. woodii*; Scatopsidae in *C. stenantha*; Fig. 2) and in four of the species they belonged to two families (e.g., Chloropidae and Milichiidae in *C. dolichophylla*, *C. nilotica*, and *C. sandersonii*; Fig. 2). Flowers of *C. denticulata* did not contain flies with pollinaria attached (Table 1). Very small flies of Cecidomyiidae, Ceratopogonidae, and from other lower Diptera were found

Table 1
Number and identity of flies collected from flowers of *Ceropegia* species in China (CN), Austria (AT), Germany (DE), Republic of South Africa (SA), Tanzania (TZ), and Burkina Faso (BF). If not indicated otherwise, all flies were female. M: male; superscript#: number of flies with pollinaria. Pollinating fly taxa are printed in bold. Pollinator data of *C. dolichophylla* and *C. sandersonii* were obtained from previous studies (Heiduk et al., 2015, 2016; see 2.1).

	C. dolichophylla	*C. sandersonii*		*C. rupicola*	*C. stenantha*		*C. ampliata*	*C. denticulata*	*C. barklyi*	*C. carnosa*		*C. haygarthii*	*C. woodii*	*C. crassifolia*	*C. nilotica*	*C. pachystelma*	*C. cycniflora*
Location code:	*CN*	*DE*	*SA*	*AT*	*DE*	*TZ*	*DE*	*DE*	*SA*	*SA*	*BF*	*SA*	*SA*	*SA*	*SA*	*SA*	*SA*
Total number of flies:	**119**	**70**	**54**	**2**	**10**	**59**	**3**	**8**	**165**	**38**	**2**	**47**	**9**	**7**	**118**	**43**	**33**
CERATOPOGONIDAE			**1**						**165**	**1**		**32**	**9**		**13**	**43**	
Forcipomyia Meigen, 1818 sp.									1M[1],86[27]	1		32	9[5]			43[16]	
unknown			1						1M,77[6]						13		
CHLOROPIDAE	**7**		**3**									**11**			**2**		**5**
Arcuator Sabrosky, 1985 **sp. nov. 1**												8[1]					
Arcuator sp.			1M[1]														1
Conioscinella Duda, 1929 sp.	2[1]																2
Gaurax Loew, 1863 sp. 1												1					
Gaurax sp. 2												1					
Polyodaspis Duda, 1933 sp.	1M[1],2[2]																
Trachysiphonella Enderlein, 1936 **sp. nov.**															2[1]		
Trachysiphonella Enderlein, 1936 sp. nov. 1			2														
Trachysiphonella sp.																	2
Tricimba Lioy, 1864 **sp. 1**	1[1]																
Tricimba sp. 2	1[1]																
Tricimba sp. nov. (*lineella* Fallén group)												1M					
DROSOPHILIDAE				**2**										**7**			
align=ëenter¨																	
Drosophila immigrans (Sturtevant, 1921)				1M[1]													
Drosophila melanogaster Meigen, 1830				1[1]													
Drosophila Fallén, 1823 sp. 1														1M,1			
Drosophila sp. 2														1M			
Drosophila sp. 3														1M[1],1[1]			
Apenthecia Tsacas, 1983 sp.														2[2]			
MILICHIIDAE	**43**	**69**	**45**					**8**							**99**		**28**
align=ëenter¨																	
Desmometopa sordida (Fallén, 1820)		6M,57[10]	1					1M,7									
Desmometopa glandulifera Brake & Freidberg, 2003															6		
Desmometopa inaurata Lamb, 1914			5												1		
Desmometopa interfrontalis Sabrosky, 1965															12[1]		
Desmometopa m-nigrum (Zetterstedt, 1848)			3														
Desmometopa microps Lamb, 1914	10M[6],13[6]	6[3]															
Desmometopa aff. **n *udigena*** Sabrosky, 1983			29[16]														
Desmometopa varipalpis Malloch, 1927	1																
Desmometopa Loew, 1866 **sp. 2**			1												79[9]		
Desmometopa sp. 3			1														
Desmometopa sp. 6 (aff. *singaporensis*)			1														
Leptometopa latipes (Meigen, 1830)																	4
Leptometopa nilssoni Sabrosky, 1987																	24[6]
Leptometopa rufifrons Becker, 1903			3														
Milichiella Giglio-Tos, 1895 sp.			1														

Table 1 (*Continued*)

	C. dolichophylla	*C. sandersonii*		*C. rupicola*	*C. stenantha*		*C. ampliata*	*C. denticulata*	*C. barklyi*	*C. carnosa*		*C. haygarthii*	*C. woodii*	*C. crassifolia*	*C. nilotica*	*C. pachystelma*	*C. cycniflora*
Location code:	*CN*	*DE*	*SA*	*AT*	*DE*	*TZ*	*DE*	*DE*	*SA*	*SA*	*BF*	*SA*	*SA*	*SA*	*SA*	*SA*	*SA*
Milichia Meigen, 1830 sp.															1		
Neophyllomyza leanderi (Hendel, 1924)	10^{7}																
Neophyllomyza Melander, 1913 sp.	9^{7}																
PHORIDAE align=ĉenter¨	**3**									**38**	**2**						
Megaselia Rondani, 1856 **sp. 1**										11^{6}							
Megaselia sp. 2										1							
Megaselia sp. 3										1^{1}							
Megaselia sp. 4										1							
Megaselia sp. 5	1									5	2						
Megaselia sp. 6										10^{5}							
Megaselia sp. 7	1																
Megaselia sp. 8										8^{3}							
Megaselia sp. 9	1																
SCATOPSIDAE			**1**		**10**	**56**											
Coboldia fuscipes (Meigen, 1830)					$1M^{1}$												
Neorhegmoclemina Cook, 1955 **sp. nov. 1 aff. *divergens*** Cook, 1965						$8M,21^{6}$											
Neorhegmoclemina sp. nov. 2 aff. *chaetophora* Cook, 1965						1M											
Psacotes sp. nov. 2			1														
Rhegmoclemina Enderlein, 1936 sp.						4^{1}											
Rhegmoclemina sp. nov. aff. *edwardsi* Cook, 1960						1M											
Rhegmoclematini Cook, 1965 indet. sp.						2											
Swammerdamella Enderlein, 1912 aff. *rutosa* Cook, 1965						5M											
Swammerdamella brevicornis (Meigen, 1830)					$7M^{6},2^{2}$												
Swammerdamella Enderlein, 1912 **sp. 1**						4^{2}											
Swammerdamella sp. nov. 1						2M											
Swammerdamella aff. **sp. nov. 2**						1^{1}											
Thripomorpha Enderlein, 1905 sp. nov. 1 aff. *fragile* (Cook, 1965)						1M											
Thripomorpha sp. nov. 2						3M,3											
ANTHOMYIIDAE							**1**										
Delia platura (Meigen, 1826)							$1M^{1}$										
MUSCIDAE							**1**										
Muscina stabulans (Fallén, 1817)							1M										
TACHINIDAE							**1**										
Blondelia nigripes (Fallén, 1810)							1^{1}										
LAUXANIIDAE												**1**					
Homoneura van der Wulp, 1891 sp.												1					
OTHER DIPTERA	**66**	**1**	**4**			**3**						**3**			**4**		
Cecidomyiidae			1			3						1M,1					
Psychodidae												1M					
Chironomidae			1														
Unknown lower Diptera	65	1															
Unknown	1		2												4		

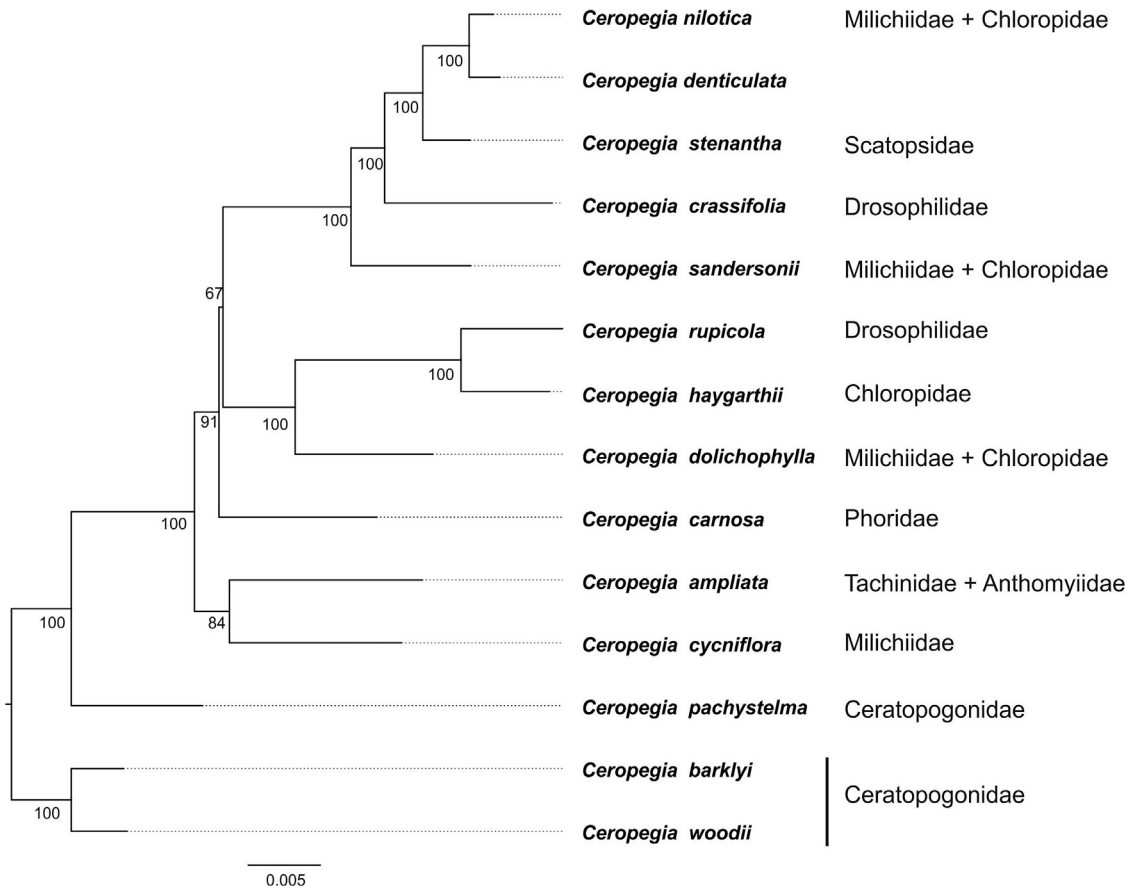

Fig. 2. Phylogenetic tree based on Maximum Likelihood analysis of six markers of nuclear and cpDNA. For all species except for *C. denticulata*, for which no data on fly pollinators are available, pollinating fly families are indicated. Numbers below branches indicate frequency of bipartitions.

in flowers of several species (Table 1), however, they only carried pollinaria when collected from *Ceropegia* species with flowers that are rather small sized with narrow tubes (*C. woodii*, *C. pachystelma*, *C. barklyi*; Table 1, Fig. 1). Most flies had one pollinarium attached, but two Drosophilidae collected from flowers of *C. crassifolia* carried two and four pollinaria, each.

For *C. stenantha*, flower visitors were collected in the native (Tanzania) and non-native (Germany) range, and flowers of both collection sites exclusively contained Scatopsidae. However, the scatopsid species differed among sites and flowers from the native range contained a greater variety of species (eleven different species, four with pollinaria attached) than flowers of greenhouse plants in the non-native range (two species, both with pollinaria).

Pollen transfer efficiency PTE was zero in *C. haygarthii* ($n_{flowers}$ = 60), 3% in *C. woodii* ($n_{flowers}$ = 36), 4% in *C. nilotica* ($n_{flowers}$ = 7), and 7% in *C. barklyi* ($n_{flowers}$ = 56), *C. carnosa* ($n_{flowers}$ = 38) and *C. crassifolia* ($n_{flowers}$ = 4). In *C. pachystelma* ($n_{flowers}$ = 61) PTE was 33%.

3.2. Flower scent

The amount of scent released from a single flower varied among species and ranged from 1 ng per 15 min in *C. pachystelma* to >1000 ng in *C. cycniflora* (Table 2). In total 317 volatiles, mainly aliphatic components, aromatic components, terpenes, and various unknown components, were detected in the headspace samples of the 14 *Ceropegia* species (Table S3). The number of floral volatiles per species varied from five in *C. woodii*, *C. crassifolia*, and *C. pachystelma* to 56 in *C. sandersonii* (Table 2). Scent composition was highly variable among species and most components occurred only in a single species (e.g., 3-acetyloxy-4-phenylbutan-2-one and 3-acetyloxy-1-phenylbutan-2-one in *C. stenantha*). The percentage of species-specific components ranged from 100% in *C. crassifolia* to 40% in *C. woodii* (Table 2). Also, relative floral scent patterns strongly differed among species (ANOSIM: R = 0.856, P < 0.001, Fig. 3A + B). With the exception of *C. sandersonii* and *C. denticulata*, which both released high relative amounts of the aliphatic components 2-heptanone and 2-nonanol (compare Table 2), each species had a different volatile as main constituent, and this volatile did not occur in any of the other investigated species (Table 2). Examples for such species-specific main volatiles are the aliphatic components (2*S*,6*R*,8*S*)-8-methyl-2-propyl-1,7-dioxaspiro[5.5]undecane, acetoin, and hexyl butyrate in *C. dolichophylla*, *C. crassifolia*, and *C. cycniflora*, respectively (Tables 2 and S3; see also Fig. 3).

3.3. Relationships between relatedness of plants, floral scent and flower visiting/pollinating flies

A comparison of relatedness among the *Ceropegia* species with their visitor/pollinator assemblages and scent chemistry revealed that some quite closely related species have similar visitors/pollinators and similar floral scents (*C. sandersonii* and *C. denticulata*; Tables 1 and 2; Figs. 2 and 3), other closely related species have similar pollinators and different scents (*C. woodii*, *and C. barklyi*; Fig. 2), and again others have different pollinators and different scents (*C. crassifolia*, *C. stenantha*, and *C. nilotica*; *C. rupicola* and *C. haygarthii*; *C. ampliata* and *C. cycniflora*; Tables 1 and 2; Figs. 2 and 3).

Overall, there were no phylogenetic signals in floral scent chemistry (K_{mult} = 0.316, p = 0.628) and flower visiting flies on morphospecies level (K_{mult} = 0.412, p = 0.093). However, we found phylogenetic signal in flower visiting flies on family level

Table 2
Mean (±SD) relative amounts of floral volatiles identified with >5% in scent samples collected from flowers of *Ceropegia* species. Total number of floral volatiles, mean total absolute amount, number of plant individuals, number of sampled flowers, and sampling time (min-max) are also given for each species. SD: Standard deviation; [S]: component verified through authentic standard; tr: amount <0.5%; values in bold: amount ≥5.0%. Scent data for *C. dolichophylla* and *C. sandersonii* were obtained from previous studies (Heiduk et al., 2015, 2016; see 2.1). *C. dol*: *C. dolichophylla*; *C. san*: *C. sandersonii*; *C. rup*: *C. rupicola*; *C. ste*: *C. stenantha*; *C. amp*: *C. ampliata*; *C. den*: *C. denticulata*; *C. bar*: *C. barklyi*; *C. car*: *C. carnosa*; *C. hay*: *C. haygarthii*; *C. woo*: *C. woodii*; *C. cra*: *C. crassifolia*; *C. nil*: *C. nilotica*; *C. pac*: *C. pachystelma*; *C. cyc*: *C. cycniflora*.

	C. dol	*C. san*	*C. rup*	*C. ste*	*C. amp*	*C. den*	*C. bar*	*C. car*	*C. hay*	*C. woo*	*C. cra*	*C. nil*	*C. pac*	*C. cyc*
Plant individuals (#flowers):	**12 (21)**	**5 (10)**	**1 (6)**[a]	**1 (3)**[a]	**>1 (6)**[a]	**>1 (9)**[b]	**6 (20)**	**7 (18)**	**1 (9)**[a]	**1 (6)**[a]	**2 (4)**	**1 (4)**[a]	**5 (15)**	**1 (3)**[a]
Sampling time [min]:	15	15–25	20–60	15	15	7	20–120	15–55	15–35	20–35	25–35	20–35	25–70	40–110
Mean total scent emitted per flower [ng/15 min ± SD]:	51 ± 31	79 ± 77	31 ± 30	204 ± 32	9 ± 9	194 ± 95	12 ± 11	3 ± 5	122 ± 93	10 ± 8	198 ± 42	108 ± 71	1 ± 1	1043 ± 878
Total # of components:	52	56	41	19	30	54	14	14	15	5	5	19	5	36
Species specific components [%]:	96	46	80	95	80	61	93	36	60	40	100	95	80	67
Compound class														
Aliphatic components														
Acetoin[S]											**51 ± 6**			
2,3-Butanediol[S]											**42 ± 11**			
2,3-Butanedion[S]											**7 ± 5**			
Decane[S]										**87 ± 30**				
Ethyl dodecanoate[S]												**83 ± 10**		
a Heptadecene							**68 ± 8**							
a Heptadecene							**15 ± 6**							
a Heptadecene							**6 ± 6**							
2-Heptanol[S]		**9 ± 2**				**14 ± 10**	tr ± tr			1 ± 2				
2-Heptanone[S]		**24 ± 14**				**63 ± 15**				1 ± 2				
(*E*)-2-Hexenal[S]								**15 ± 19**						2 ± 2
(*Z*)-3-Hexen-1-ol[S]			tr ± tr		**11 ± 10**			1 ± 3						tr ± tr
(*E*)-2-Hexenyl acetate		tr ± tr												**13 ± 18**
(*Z*)-3-Hexenyl acetate[S]		2 ± 3	1 ± 1		**16 ± 10**	tr ± tr		**17 ± 34**	3 ± 2					tr ± tr
(*E*)-2-Hexenyl butyrate[S]														**11 ± 8**
Hexyl butyrate[S]		tr ± tr												**46 ± 10**
Isobutyl acetate			**21 ± 23**											
2-Nonanol[S]		**25 ± 16**				**9 ± 9**								
2-Nonyl acetate[S]		**11 ± 8**				**tr ± tr**								
(*E*)-2-Octenyl acetate[S]		**7 ± 7**				tr ± tr								tr ± tr
(*E*)-2-Octenyl butyrate		tr ± tr												**11 ± 9**
5-Pentadecene	2 ± **5**													
6- + 7-Pentadecene	**6 ± 6**													
a Pentenal								**9 ± 21**						

Tridecane[S]	**22 ± 16**											
Undecane[S]	3 ± **6**											
C5-Branched chain components												
3-Methylbutan-1-ol[S]			3 ± 4				**7 ± 18**					
3-Methyl-2-butenyl acetate[S]		1 ± tr							4 ± **9**			
3-Methyl-3-butenyl acetate									**7 ± 16**			
Aromatic components												
Benzaldehyde[S]				**65 ± 15**								
a Methyl methylsalicylate								**85 ± 6**				
Methyl phenylacetate[S]					**55 ± 10**							
1-Phenyl-2,3-butanedione[S]				**23 ± 12**								
2-Phenylethanol[S]				tr ± tr	4 ± **6**	tr ± tr		1 ± 1				
Terpenes												
Monoterpenes												
β-Citronellol[S]								**6** ± 3				
Limonene[S]										**6 ± 7**		
Linalool[S]		2 ± 3	1 ± 1		4 ± **6**			1 ± tr				
(*E*)-β-Ocimene[S]		1 ± 1			2 ± 3	tr ± tr	**14 ± 35**	1 ± 1				
Sesquiterpenes												
allo-Aromadendrene											**9** ± 2	
β-Bourbonene			tr ± tr				**5 ± 12**					
N-containing components												
2-Methoxy-3-isopropyl-pyrazine										3 ± **5**		
N-(3-Methylbutyl)acetamide[S]	**11 ± 13**											
Spiroacetals												
(*E,E*)-2,8-Diethyl-1,7-dioxaspiro[5.5]undecane[S]	**8 ± 4**											
(*E,E*)-2,8-Dimethyl-1,7-dioxaspiro[5.5]undecane[S]	**5 ± 5**											
(2*S*,6*R*,8*S*)-8-Methyl-2-propyl-1,7-dioxaspiro[5.5]undecane[S]	**30 ± 17**											
Unknown components												
m/z: 95, 110, 67, 43			**46 ± 24**									
m/z: 57, 43, 71, 85, 99, 212						3 ± **5**						
m/z: 120, 138							**29 ± 45**					
m/z: 189, 204, 41, 91, 119											**84** ± 3	

[a] Mean values calculated across samples from different flowers of same individual.

[b] Mean values calculated across samples from different flowers not assigned to plant individuals.

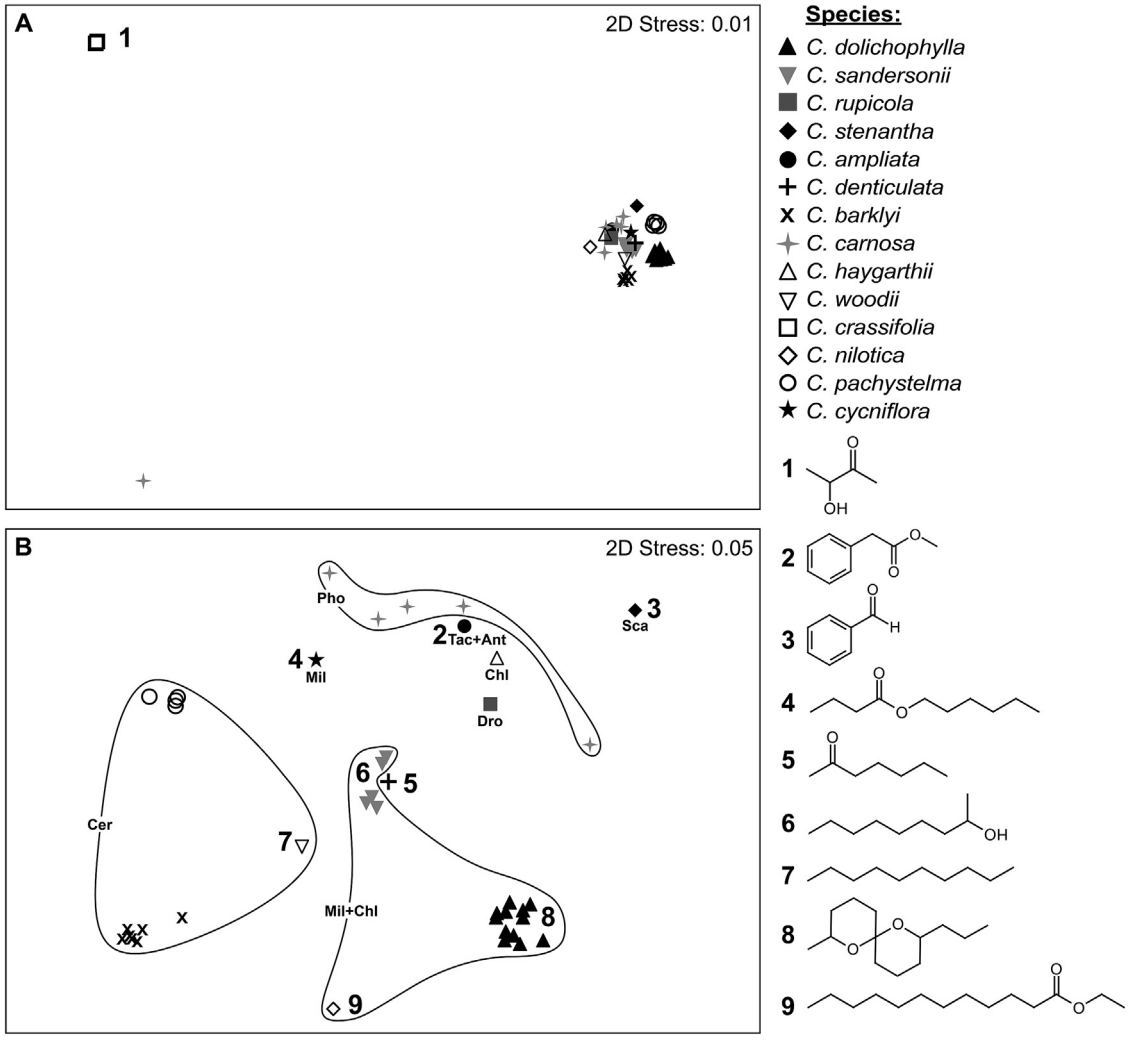

Fig. 3. Non-metric multidimensional scaling (NMDS) of scent samples collected from different *Ceropegia* species based on semi-quantitative Bray-Curtis similarities. **A:** NMDS including all samples from the investigated species. **B:** NMDS excluding *C. crassifolia* and two outlier samples of *C. carnosa*. Chemical structures of identified main scent components are given as **1:** acetoin; **2:** methyl phenylacetate; **3:** benzaldehyde; **4:** hexyl butyrate; **5:** 2-heptanone; **6:** 2-nonanol; **7:** decane; **8:** (2*S*,6*R*,8*S*)-8-methyl-2-propyl-1,7-dioxaspiro[5.5]undecane; **9:** ethyl dodecanoate. Pollinating fly taxa are indicated as Ant: Anthomyiidae; Cer: Ceratopogonidae; Chl: Chloropidae; Dro: Drosophilidae; Mil: Milichiidae; Pho: Phoridae; Sca: Scatopsidae; Tac: Tachinidae.

(K_{mult} = 0.736, p = 0.003) and in assemblages of pollinating flies used by the *Ceropegia* species (family level: K_{mult} = 0.725, p = 0.019; morphospecies level: K_{mult} = 0.749, p = 0.003).

Floral scent patterns did not correlate with flower visiting flies (RELATE: family level, Rho = 0.025, p = 0.42; morphospecies level, Rho = −0.006, p = 0.50) nor with fly pollinators (RELATE: family level, Rho = 0.041, p = 0.40; morphospecies, Rho = −0.013, p = 0.56).

4. Discussion

Our data revealed that (1) *Ceropegia* species are generally specialized on small flies of only one or two families/genera as pollinators and have a low reproductive success, (2) floral scents are highly variable among species, (3) there is a phylogenetic signal in flower visiting fly families and pollinator assemblages, but flower visiting fly morphospecies and floral scent chemistry are not linked to the phylogeny of plants, and (4) overall floral scent chemistry does not correlate with pollinator assemblages.

4.1. Flower visitors, pollinators and pollen transfer efficiency (PTE)

In agreement with the results of previous studies (Coombs et al., 2011; Heiduk et al., 2010; Karuppusamy and Pullaiah, 2009; Masinde, 2004; Ollerton et al., 2009; Vogel, 1961) the investigated *Ceropegia* species are pollinated exclusively by Diptera <3 mm (only in *C. ampliata* >3 mm) of several families. Most of these families have also been reported as pollinators in other asclepiads (Nihei and Schwarz, 2011; Ollerton et al., 2003; Ollerton and Liede, 1997, 2016; Yassin et al., 2012) and/or other plant families (see e.g., Corlett, 2004; Larson et al., 2001; Orford et al., 2015). Thirty three fly morphospecies were identified as pollinators in the present study, and most of these taxa were not known as pollinators of *Ceropegia* before (Ollerton et al., 2009). Ten of the 18 genera documented here are reported as pollinators in *Ceropegia* for the first time. With the exception of Anthomyiidae, the observed fly families, however, are already known to be pollinators of the same or other *Ceropegia* species inside and outside their native ranges (Bhatnagar, 1986; Coombs et al., 2011; Ollerton and Forster, 1995; Ollerton et al., 2009; Vogel, 1961). Our data on pollinating flies of *C. carnosa*, *C. haygarthii*, *C. nilotica*, and *C. stenantha* are at least partly consistent with those published previously for these species (Coombs et al., 2011; Ollerton et al., 2009; including Supplemental Data). For the other *Ceropegia* species investigated here, the flower visitors and pollinators have not been reported before.

Interestingly, closely related *Ceropegia* species (Fig. 2; see also Bruyns et al., 2015) had similar pollinator assemblages at the fam-

ily level, as indicated by a K_{mult} analysis. Such a phylogenetic signal in plant-pollinator associations has also been shown in deceptive orchids of the genus *Chiloglottis* (Mant et al., 2002). Despite this more general phylogenetic signal in *Ceropegia*, we also found some closely related species (Fig. 2; see also Bruyns et al., 2015) associated with a different pollinator assemblage (*C. stenantha*, *C. crassifolia*, and *C. nilotica*; *C. rupicola* and *C. haygarthii*; Fig. 2).

The species studied here are functionally highly specialized on flies of only one or two families, or even genera. Similar trends have been shown for other species of *Ceropegia* by Ollerton et al. (2009). However, the ecological specialization is less distinctive since the functional pollinator group often comprised several species. The highest variety of flower visitors with 12 fly species (only native range) was found in *C. stenantha*. All but three flies were Scatopsidae of four genera, and flies of four species in three scatopsid genera carried pollinaria.

In *C. stenantha* and in *C. sandersonii* we recorded data on flower visitors and pollinators in both native (South Africa, Tanzania) and non-native ranges (Europe), however, the pollinating families are the same and even the genera are similar in both ranges (Table 1). Furthermore, for *C. ampliata* and *C. denticulata* our flower visitor/pollinator data from non-natural ranges (Europe) are consistent with published flower visitors/pollinators collected from plants within their natural range (Coombs et al., 2011; Ollerton et al., 2009). Along these lines, Ollerton et al. (2009) found that native and non-native pollinating taxa of *C. linearis* and *C. stapeliiformis* were similar. It is therefore possible that for *C. rupicola* Drosophilidae, found as the pollinators in non-native ranges, are also the pollinators in the native range of the species (Arabian Peninsula). This consistency in pollinator spectra over large geographic regions, and the finding that the species are functionally but not ecologically highly specialized suggest that the plants (plant signals) evolved to target at functional groups of pollinators and less at specific species. An exception might be *C. nilotica*, and *C. sandersonii*. Both species occur syntopically and attracted mainly different species of milichiids.

Except for *C. pachystelma*, the PTE of investigated species was low and this finding is consistent with data published for *C. dolichophylla* (Heiduk et al., 2015) and *C. ampliata* (Coombs et al., 2011). In *C. pachystelma* the number of simultaneously open flowers was much higher than in other species, which might explain our finding of high PTE (33%) in this species.

4.2. Floral scent

Ceropegia produces a mixture of widespread and highly uncommon components. For instance, linalool, (*E*)-ß-ocimene, benzaldehyde, and 2-phenylethanol haven been reported in the floral scent of many flowering plants (Knudsen et al., 2006). Whereas floral volatiles, such as 2-nonyl acetate (listed as 2-acetoxynonane in Heiduk at al., 2016), (*E*)-2-octenyl acetate, (2*S*,6*R*,8*S*)-8-methyl-2-propyl-1,7-dioxaspiro[5.5]undecane, and N-(3-methylbutyl)acetamide are not known to occur in plants others than *Ceropegia* (see Knudsen et al., 2006). The aromatic components 3-acetyloxy-4-phenylbutan-2-one and 3-acetyloxy-1-phenylbutan-2-one (Table S3) have, to the best of our knowledge, not been reported from nature before. Ongoing bioassays will proof whether or not these volatiles are important for attraction of the scatopsid pollinators. Results including details on identification and synthesis of these components will be processed in a future study.

Intraspecific variability was generally low in species for which more individuals were tested (Fig. 3A + B). However, in *C. carnosa* the intraspecific variability was particularly high (Fig. 3A + B). The collected scent samples of this species were relatively weak with low absolute amounts of scent (Table 2). It is therefore possible that we might have missed the time when the plant had its peak scent emission, which might be short (see Vogel, 1961) in this species.

Scents strongly differed among species, and these differences were independent of relatedness of the study plants. There was no phylogenetic signal in scents showing the evolutionary flexibility of this trait (for a discussion on this topic see Feulner et al., 2014; Prieto-Benítez et al., 2016; and references therein). The closely related species *C. crassifolia*, *C. denticulata*, and *C. stenantha*, emit quite different scent patterns with clear differences in main floral volatiles (*C. crassifolia*: acetoin; *C. nilotica*: ethyl dodecanoate; *C. stenantha*: benzaldehyde).

4.3. Pollinator specificity through floral scent

Our data show that pollination systems in *Ceropegia* are highly specific and we assume that this specificity is due to differential scent chemistry, even though floral scent did not correlate with pollinator assemblages, neither on family nor on morphospecies level. The missing correlation in our analyses is probably due to the fact that there was limited overlap in scent and pollinators among species, and most scent components and pollinator taxa were species specific. Further, some plants, such as *C. sandersonii* and *C. dolichophylla*, attract the same functional group of pollinators (*Desmometopa* spp.) with different scent components. Thus, despite these species have different scent chemistry, their floral odors address the same fly group. Both *Ceropegia* species were shown or believed to be kleptomyiophilous and mimic the food of their kleptoparasitic *Desmometopa* pollinators, i.e. attacked honey bees (*C. sandersonii*; Heiduk et al., 2016) and paper wasps (*C. dolichophylla*; Heiduk et al., 2015). They exploit different olfactory preferences of the same flies. Also, our analyses were performed with all components and not only those involved in pollinator attraction. Results may differ when including only biologically active components in the analyses (see also discussion in Shuttleworth and Johnson, 2012).

Ceropegia sandersonii and *C. dolichophylla* are the only species with known/suggested pollination strategy (see above), and data recorded for pollinators and floral scents in the present study allow speculating about the deceptive strategy of other species. Floral scent of *C. denticulata* is very similar to that of *C. sandersonii* and it is also visited by kleptoparasitic *Desmometopa* flies. We therefore assume that *C. denticulata* has the same pollination strategy as *C. sandersonii* and also mimics volatiles released by honeybees attacked by an arthropod predator. Flowers of *C. cycniflora* were also pollinated by kleptoparasitic Milichiidae and main floral scent components were hexyl butyrate, (*E*)-2-hexenyl acetate, (*E*)-2-hexenyl butyrate, and (*E*)-2-octenyl butyrate, all well known as defensive secretions of true bugs (e.g., Aldrich et al., 1999; see also El-Sayed, 2014; Ho and Millar, 2002; Oelschlägel et al., 2015). True bugs are a common food source for kleptoparasitic flies, including flower visitors and pollinators of *C. cycniflora*, and these flies are attracted to the defensive volatiles released by the bugs when attacked or injured (Kondo et al., 2011; Oelschlägel et al., 2015; Zhang and Aldrich, 2004). Thus, for *C. cycniflora* a kleptomyiophilous pollination strategy is also very likely, and defending true bugs could well be the model mimicked with the floral scent, as was recently described for a deceptive *Aristolochia* species (Oelschlägel et al., 2015). Also chloropid flies, identified as pollinators of *C. haygarthii*, are attracted by defensive secretions of true bugs (e.g., Oelschlägel et al., 2015). Flowers of this plant release mainly a methyl methylsalicylate, a component not known from true bugs so far. It remains unclear whether this plant is pollinated by chloropids looking for food or exploits another behavior of the flies.

Ceropegia nilotica, like *C. sandersonii*, *C. denticulata*, *C. dolichophylla*, and *C. cycniflora*, is also pollinated by kleptoparasitic milichiid flies, and the main floral component is ethyl dode-

canoate. This component is described as pheromone component of different hymenopterans (Coppée et al., 2008; Kullenberg et al., 1970; Leonhardt et al., 2009), potential candidates mimicked by *C. nilotica*. Further experiments are needed to test this hypothesis.

Ceropegia carnosa is pollinated by Phoridae of the genus *Megaselia*. Adults of phorids, including *Megaselia*, are ecologically highly diverse. Some are parasitoids (Dutto and Ferrazzi, 2014; Gonzalez et al., 2002; Menail et al., 2016), others specialist predators, saprophages or kleptoparasites (Disney, 1994; Disney and Fayle, 2008; Hash, 2014). Floral scent of *C. carnosa* contained (*E*)-2-hexenal and (*Z*)-3-hexenyl acetate, both widespread green leaf volatiles (e.g., Ruther, 2000). Interestingly these volatiles are also known as secretions of true bugs (Aldrich et al., 1993; see also El-Sayed, 2014; Marques et al., 2000; Sachin et al., 2008). Although speculative, kleptomyiophily seems to be a possible pollination strategy of *C. carnosa*.

Flowers of *Ceropegia crassifolia* are pollinated by drosophilid flies, and the floral scent of this species was dominated by acetoin, 2,3-butanediol, and 2,3-butanedione. These components are known to be perceived by drosophilids (Cha et al., 2014; De Bruyne et al., 2001; Stensmyr et al., 2003), and acetoin in particular has been shown to be an attractant for such flies (Cha et al., 2014). Drosophilids feed on and breed in several kinds of (over)ripe fermenting fruit (Walsh et al., 2011). Acetoin, 2,3-butanediol, and 2,3-butanedione are yeast-produced chemicals associated with fermentation processes (Magee and Kosaric, 1987) and released from fermenting fruits (Stökl et al., 2010). Thus, it is likely that *C. crassifolia* targets its drosophilid pollinators through brood site and/or food source mimicry, a strategy also used by a deceptive *Arum* species (Stökl et al., 2010). *Ceropegia rupicola* is also pollinated by Drosophilidae, and we believe that flower scent of this species also tragets drosophilids by mimicry of fermenting fruit. Unfortunately, the main floral scent component could not be identified by us. However, two volatiles, isobutyl acetate and 3-methyl-1-butanol could be involved in attracting drosophilids. Isobutyl acetate is produced by yeasts (Becher et al., 2012; Buzzini et al., 2003), and both components are described from odor of different fruits (Jordán et al., 2001; Shalit et al., 2001; Zabetakis and Holden, 1997) and are biologically active in *Drosophila* (Becher et al., 2012; Schubert et al., 2014).

Flowers of *Ceropegia stenantha* are exclusively pollinated by Scatopsidae, known as scavengers or detritivores (Freeman, 1985; Haenni, 1997; Haenni and Vaillant, 1994). These flies are likely attracted by decaying leaf litter as it starts fermenting to feed on the liquified substrate (Perez et al., 2013). Scatopsidae have also been reported as flower visitors feeding on nectar and pollen (García-Robledo and Mora, 2007; Larson et al., 2001; Woodcock et al., 2014). However, little is known about the chemical ecology of these flies (El-Sayed, 2014). We do not consider benzaldehyde as key attractant because benzaldehyde is a very widespread scent component in angiosperms emitted by flowers of many different plant groups that are not associated with Scatopsidae (El-Sayed, 2014; Knudsen et al., 2006). The second most abundant component 1-phenyl-2,3-butanedione has been rarely described as a floral volatile (Joulain, 1987; Wong and Teng, 1994), and we do not know whether scatopsid flies are attracted by this component. Another possibility is that the new natural components identified by us in the floral scent of *C. stenantha* are specific attractants for Scatopsidae. Interestingly, a scatopsid species of a genus not occurring as visitor of *Ceropegia* is attracted to 4-(*p*-acetoxyphenyl)-butan-2-one (Uchida et al., 2003), a component structurally related to the new natural components.

Ceropegia pachystelma, *C. barklyi*, and *C. woodii* are pollinated by the ceratopogonid fly genus *Forcipomyia*, and the identified floral components are hydrocarbons (i.e., decane, heptadecenes). *Forcipomyia* flies are known to breed in leaf-litter (Winder and Silva, 1972) and adult flies are (klepto)parasites and blood suckers (Downes, 1958; Glukhova, 1989; Marshall et al., 2015; Martens et al., 2008 Glukhova, 1989; Marshall et al., 2015; Martens et al., 2008). The connection between the biology of *Forcipomyia* and the flower scent of *C. pachystelma*, *C. barklyi*, and *C. woodii* is unclear and the pollination strategy of these *Ceropegia* species remains unknown.

Floral scent of *Ceropegia ampliata* was dominated by methyl phenylacetate. However, the role of this scent component for attracting the relatively large fly pollinators of this species (see 3.1, and Coombs et al., 2011) remains unclear.

5. Conclusion

Our study contributes to the understanding of the pollination biology and scent chemistry in *Ceropegia*, and to the chemical communication between deceptive plants and their pollinators. The comparative analyses highlighted that *Ceropegia* pollination systems not only comprise kleptomyiophily, as described recently for the genus, but also other deceptive strategies such as brood site mimicry systems. The identification of novel natural components is puzzling and calls for ongoing studies to identify the biological function of these components in the biology of the deceived flies, and to identify their importance in the deceptive system of the plants.

Acknowledgements

The authors thank Hans-Peter Tschorsnig and Wolfgang Adaschkiewitz for identification of flower visitors/pollinators of *C. ampliata*, and Gerhard Baechli for identification of drosophilids from *C. rupicola* flowers. We also thank Adam Shuttleworth, David Styles, Taina Witt, and Irmgard Schäffler for help during field trips. We further thank Robert von Blittersdorf† for providing alcohol material of *C. stenantha* flowers collected in Tanzania. The authors also thank Adjima Thiombiano, Stefan Porembski and Christoph Höpel who kindly provided alcohol material of *C. carnosa* collected in Bukina Faso during field trips for the BMBF program WASCAL. The research was funded by a grant for PhD candidates according to the Bavarian Elite Aid Act (BayEFG).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.flora.2017.02.001.

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Heiduk, A., Brake, I., von Tschirnhaus, M., Haenni, J.-P., Miller, R., Hash, J., Prieto-Benítez, S., Jürgens, A., Johnson, Steven D., Schulz, S., Liede-Schumann, S., Meve, U., & Dötterl, S. (2017): Floral scent and pollinators of *Ceropegia* trap flowers. Flora 232, 169-182. **(Publication 3 of this thesis)**

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Heiduk, A., Kong, H., Brake, I., von Tschirnhaus, M., Tolasch, T., Tröger, A.G., Wittenberg, E., Francke, W., Meve, U. & Dötterl, S. (2015): Deceptive *Ceropegia dolichophylla* fools its kleptoparasitic fly pollinators with exceptional floral scent. Frontiers in Ecology and Evolution 3: 66. doi: 10.3389/fevo.2015.00066. **(Publication 1 of this thesis)**

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Acknowledgments

I would like to express my special appreciation and thanks to my advisors **Ulrich Meve** and **Stefan Dötterl**, you have been brilliant mentors for me. I would like to thank you for introducing me to *Ceropegia* and for the opportunity to work on the pollination biology of these fascinating plants. You gave me invaluable guidance and constant support. You have been optimistic all the time and dissipated the many doubts I often had. Thank you for motivating and always encouraging me, and for allowing me to grow as a research scientist.

Besides my mentors, who initiated my PhD thesis, I would further like to express my gratitude to all those people, without whom my research would not have been possible.

First of all I am grateful to **Andreas Jürgens** and **Taina Witt**. Thanks to you my research trips to South Africa were beyond compare. I lack the words to express how much I appreciate what you did for me. Besides providing me your outstanding scientific support, you always made me feel more than welcome. I am so deeply thankful.

I further want to thank **Steven D. Johnson** for providing me his lab facilities at the University of KwaZulu-Natal in Pietermaritzburg, and for valuable discussions especially regarding my publications.

I am also truly thankful to **Adam Shuttleworth**, **David Styles**, and **Neil Crouch** for the many fieldtrips to marvelous habitats of my study plants, and for indispensable botanical knowledge. During our trips, I always had a great time with lots of fun.

I especially want to mention **Irmgard Schäffler**, who not only accompanied me on field trips to South Africa and China, but who also assisted me in manifold ways. You always selflessly and unconditionally stood by my side when things were complicated. To me, your friendship is of inestimable value.

There are many more people whose help I obtained and all of whom contributed to my thesis. Among them **Sigrid Liede-Schumann**, who always had an open door and ear, gave great advice and words of comfort, and had great faith in me. I also want to thankfully list the fly taxonomists **Irina Brake**, **Michael von Tschirnhaus**, **Frank Menzel**, **John Hash**, **Jean-Paul Haenni**, **Raymond Miller**, **Hans-Peter Tschorsnig**, **Wolfgang Adaschkiewitz**, **Susanne Prescher**, and **Gerhard Bächli** who all together did a very great job. Further to mention are **Hanghui Kong**, who performed bioassays and enabled my research trip to China, and **Yu Hong** who was my personal guide in China. There should be listed several more people here such as students who collected useful data during practical courses. To all those, whose names are not given: Indulge me! I markedly appreciate what you did for me.

Last and most importantly, I am deeply grateful to **my parents**. The values you have taught me were of great help to follow through my PhD and to never ever give up on life. I love you!

This project gave me the chance to travel abroad and to take part in great national and international conferences. The research trips to South Africa and China as well as the participation in conferences would not have been possible without the financial support from the Bavarian Elite Aid Act (BayEFG).
During my PhD I gained many unforgettable experiences, and met awesome people and excellent scientist. Unfortunately, I did not have the chance to meet **Stefan Vogel**, the leading pioneer of research on *Ceropegia* pollination. With dignity I dedicate my PhD thesis to him.

Appendix

Available online at www.sciencedirect.com
ScienceDirect
South African Journal of Botany 76 (2010) 762 – 769
SOUTH AFRICAN JOURNAL OF BOTANY
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Scent chemistry and pollinator attraction in the deceptive trap flowers of *Ceropegia dolichophylla*

A. Heiduk [a], I. Brake [b], T. Tolasch [c], J. Frank [d], A. Jürgens [e], U. Meve [a], S. Dötterl [a,*]

[a] *Department of Plant Systematics, University of Bayreuth, Universitätsstr. 30, 95440 Bayreuth, Germany*
[b] *Entomology Department, Entomology Faunas and Floras, Natural History Museum, Cromwell Road SW7 5BD, London*
[c] *Tierökologie, AG Pheromone, Universität Hohenheim, Garbenstr. 30, BIO II, 70599 Stuttgart, Germany*
[d] *Bergstr. 12, 95694 Mehlmeisel, Germany*
[e] *School of Biological and Conservation Sciences, University of KwaZulu-Natal Pietermaritzburg, Private Bag X01, Scottsville 3209, South Africa*

Received 26 May 2010; received in revised form 27 July 2010; accepted 28 July 2010

Abstract

Ceropegia species (Apocynaceae, Asclepiadoideae) have pitfall flowers and are pollinated by small flies through deception. It has been suggested that these flies are attracted by floral scent. However, the scent that is emitted from *Ceropegia* flowers has not been studied using headspace and gas chromatography mass spectrometry methods. It has also been unclear whether or not the flowers are mimics of particular models that attract flies. In the present study, we determined the composition as well as the spatial and temporal patterns of floral scent emitted by *C. dolichophylla*. Furthermore, we determined the pollinators in the native (China) and non-native (Germany) range of this species, and tested the capability of the floral scent to attract flies in the non-native range. Our data demonstrate that the floral scent, which is emitted from morning until evening, primarily from the tips of the corolla lobes, consists mainly of spiroacetals and aliphatic compounds. Milichiid flies were common visitors/pollinators in the native as well as non-native range, and were attracted by floral scent in bioassays performed in the non-native range. The compounds emitted by *C. dolichophylla* are unusual for flowers, but are well known from insect pheromones and occur in the glandular secretions of insects. The milichiid flies that visit and pollinate the flowers are kleptoparasites that feed on the prey (haemolymph or other secretions) of predatory arthropods, e.g. spiders, to which they are attracted by scent. Our data thus suggest that the floral scent of *C. dolichophylla* mimics the feeding sites of kleptoparasitic flies.

Keywords: Bioassay; Floral scent; Fly-pollination; Headspace GC–MS; Kleptoparasites; Milichiidae; Spiroacetals

1. Introduction

Plants advertise their flowers by visual (e.g. shape and colour) and olfactory (scent) cues (Chittka and Thompson, 2001), however, the specific cues (e.g. scent compounds) responsible for attraction of pollinators are understood for just a few pollination systems (e.g. Dötterl et al., 2006; Schiestl et al., 1999). In general, the olfactory display of flowers is considered to be more specific than the visual one (Dobson, 1994). Attraction of specific pollinators in specialized systems can depend on the intensity, composition and emission time of scent (Raguso, 2008).

In the present paper we describe the chemistry of floral scent in a *Ceropegia* L. species (Apocynaceae, Asclepiadoideae) and its role in attraction of pollinators. *Ceropegia* comprises more than 180 species, all restricted to the Old World. The plants are found in tropical and subtropical habitats from Canary Islands and West-Africa as far as Australia, with main distribution areas in East-Africa, India, Madagascar and China (Meve and Liede-Schumann, 2007). Characteristic for all *Ceropegia* species is their floral Bauplan of so called pitfall flowers which can assume astonishing forms and functions. The corolla of *Ceropegia* flowers is fused resulting in a basally inflated tube. The corolla lobes are fused at their tips forming a cage like

* Corresponding author. Tel.: +49 921 552466; fax: +49 921 552786.
E-mail address: stefan.doetterl@uni-bayreuth.de (S. Dötterl).

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doi:10.1016/j.sajb.2010.07.022

structure which restricts access to the flower (Vogel, 1961). The great variety in shape, size, colour, ornamentation and scent has attracted the attention of biologists for a long time (e.g. Vogel, 1961). All pollinators identified thus far are small dipterans (<3 mm in length) which belong to at least 26 genera in 20 families (Ollerton et al., 2009). The complicated pollination process, which has been described in detail by Vogel (1961), starts with the landing of the fly pollinator on the flower tip. From there the insect plunges into the slippery tube and finally slides into the inflated base. Escape from there is prevented by the presence of hairs forming a barrier between the tube and its inflated base. While being trapped within the flower for about 24 h, the fly explores its jail (for food) and comes in touch with the gynostegium, a structure formed by the fused androecium and gynoecium. The pollinaria, two discrete pollen masses (pollinia) interconnected by a mechanical clip (i.e. the corpusculum), consequently become attached to the mouthparts of the fly. If the fly carried pollinaria from a previous flower visit, one or more pollinia can be inserted into the five guide-rails on the flanks of the gynostegium. In *Ceropegia*, anthesis lasts between one to five days (Vogel, 1961; own obs.). As it withers, the flower turns downwards, obtaining at least a horizontal position (Vogel, 1961). During this process, the hairs blocking the way out of the inflated base of the tube collapse and the fly can escape. Though the flowers produce a small amount of nectar, they are considered deceptive flowers (Vogel, 1961, 1993). This is because the primary reason for flies to visit the flowers is unlikely to be the small amount of nectar they contain. The majority of fly species that visit flowers of *Ceropegia* feed either in the larval or adult stage on animals or animal secretions, and find these food sources using odour cues (Vogel, 1961, 1993). *Ceropegia* may therefore mimic animal-related odours, though other possibilities are mimicry of rotting plant material, because it is used as food substrate by larvae of some flies, and mimicry of male sex pheromones, because flies attracted are mostly female (Ollerton et al., 2009; Vogel, 1961). To date, odour of *Ceropegia* flowers, though discernable to the human nose, has not been analysed with modern analytical techniques, and the compounds emitted are thus unknown. Vogel (1961) suggested that scent is emitted from the distal corolla lobes of the flowers. The period of scent emission begins at anthesis and lasts, depending on species, for a few hours to a few days (Vogel, 1961). Interactions between *Ceropegia* flowers and flies have been assumed to be mediated by floral scent (Vogel, 1961). Indeed, observations and experiments conducted in the lab point towards a function of floral scent for attracting flies from a distance and also for eliciting landing behaviours. Visual cues may play a secondary role in short-distance attraction (Vogel, 1961).

Ceropegia dolichophylla Schltr., the subject of this paper, is native to South China. We have cultivated a few individuals of this plant since 2007 in a greenhouse in Bayreuth. These plants regularly produce fruits with fertile seeds indicating that there are insects successfully transferring pollinia in the greenhouse.

As a first step to understanding the pollination systems in *Ceropegia*, we determined the pollinators of *C. dolichophylla*, and analysed its floral scents. We specifically asked, 1) which flies are pollinators/flower visitors in the native range in China, and in the greenhouse in Bayreuth? 2) which scent compounds are emitted by the flowers? 3) what is the temporal and spatial pattern of scent emission? and 4) is scent responsible for attraction of flies in the non-native range?

2. Methods and materials

2.1. Flower visitors and pollinators

To get information about the flower visitors and pollinators of *C. dolichophylla* in its native range, 100 flowers were collected in a natural habitat in the Chinese province of Guizhou on 9th July 2008 (UBT, for voucher details see Plant material). Picked flowers were immediately transferred into ethanol and subsequently dried before shipment to Bayreuth for further investigation. In Bayreuth, flowers were opened carefully and every fly present therein was classified as far as possible, and analysed for the presence of pollinaria.

To identify the flies visiting and pollinating the flowers in a greenhouse of the University of Bayreuth, we collected 100 flies inside the flowers during summers of 2007 and 2008, determined them to genus level, and 23 thereof to species level. We also checked these 23 flies for the presence of pollinaria. The abundance and occurrence of flies strongly varied during summer, and we did not determine the proportion of flowers that contained flies or that were pollinated.

2.2. Plant material

All investigations in the non-native range (scent, flower visiting flies) are based on only one accession: China, Guizhou, Fanjing Mt. (27° 55′ N, 108° 47′ E), 7th October 2007, *Y. Zhou* sub *H. Kong* 0674, (UBT). Living plants were raised from seeds collected at the original locality and grown in the greenhouse of the Dept. of Plant Systematics, University of Bayreuth.

2.3. Volatile collection

Floral volatiles were collected from cultivated *Ceropegia dolichophylla* (Fig. 1A) during daytime using dynamic headspace methods (Dötterl et al., 2005). For that purpose, individual, newly opened flowers were enclosed in polyester oven bags (5 cm × 6 cm, Toppits®, Germany) and their emitted scent was trapped by sucking the air from the bag into an adsorbent tube. Two different types of tubes were used. One type, the small sized tube, was made of ChromatoProbe quartz microvials of Varion Inc. (length: 15 mm, inner diameter: 2 mm), from which the closed end was cut off. They were filled with a mixture of 1.5 mg Tenax-TA (mesh 60–80) and 1.5 mg Carbotrap (mesh 20–40), which was fixed by using glass wool. The other and bigger type of tubes consisted of glass capillaries (length: 8 cm, inner diameter: 2.5 mm) filled with 15 mg Tenax-TA (mesh 60–80) and 15 mg Carbotrap (mesh 20–40).

The air was sucked through the tubes using a membrane pump (G12/01 EB, Rietschle Thomas Inc., Puchheim, Germany) driven by a power supply; the flow rate was adjusted to 200 ml/min

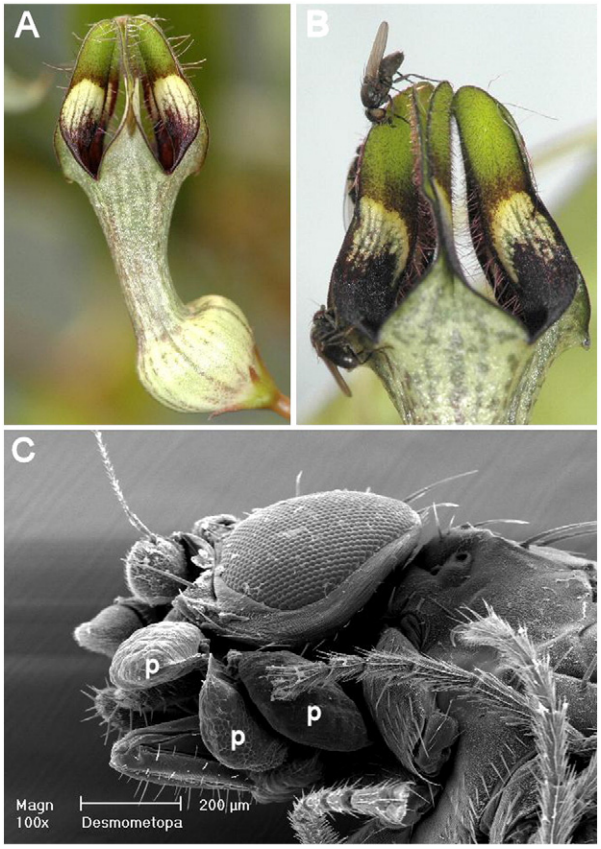

Fig. 1. (A) Flower of *Ceropegia dolichophylla*. (B) Flower tip of *C. dolichophylla* with individuals of the milichiid fly *Desmometopa sordida*. After landing, the flies crawl around on the corolla lobes extend their proboscis and probe the surface. (C) SEM of a head of *Desmometopa sordida* with pollinaria of *C. dolichophylla* attached to the base (rostrum) of its mouth parts. p = pollinium. The fly carries two pollinaria with three pollinia indicating that one pollinium was already successfully inserted into the stigmatic chamber.

(small tubes) or 100 ml/min (bigger tubes) by the use of a flow meter. To distinguish between floral and ambient air compounds, the surrounding air was collected simultaneously.

To determine the scent emitted by the flowers, individual flowers were enclosed *in situ* in the bags for 5 min followed by 2 min of scent collection into the small tubes. To analyse the spatial pattern of scent emission, five individual flowers were removed from two different plants, and cut into the four pieces 'corolla lobe tips', 'corolla lobe bases', 'corolla tube' and 'basal inflation'. For each flower these parts were enclosed separately in bags (4 cm × 5 cm) for 10 min and scent was subsequently collected for 2 min, again into the small adsorbent tubes. For the analysis of temporal scent emission, six individual flowers (two and four from different plant individuals, respectively) were separately enclosed *in situ* in oven bags for 9 h from 9 am to 6 pm, and the air was constantly sucked out using the same pumps as described above. Every hour the pumps were shut off for 10 min to allow accumulation of the floral scent, which was subsequently trapped into small adsorbent tubes for 2 min. The percentage amount of compounds was similar among the samples collected at different times (Heiduk and Dötterl, unpubl. data), and here we focus only on the total amount of scent.

To get a scent sample used for the bioassays (see below), we again enclosed individual flowers *in situ* in separate oven bags as described above. The scent was trapped using the larger adsorbent tubes and the air was sucked through the tubes for 7 h during daytime. The trapped volatiles were eluted from each adsorbent tube with 60 µl of acetone (SupraSolv, Merck KgaA, Germany). In total, we collected scent from 11 flowers, and all samples were pooled.

2.4. Chemical analysis

The volatile samples were analysed by GC–MS using a Varian Saturn 3800 gas chromatograph (GC) and a Varian Saturn 2000 mass spectrometer (MS). The GC was fitted with a 1079 injector and a ZB-5 column (5% phenyl polysiloxane, length 60 m, inner diameter 0.25 mm, film thickness 0.25 µm, Phenomenex). To allow thermal desorption of the volatiles trapped in the quartz microvials, the injector was fitted with the ChromatoProbe kit (Micro-SPE, Amirav and Dagan, 1997; see also Dötterl et al., 2005).

To flush any air from the system, the injector split vent was opened and the injector heated at 40 °C for 2 min. Then the split vent was closed, the injector heated at 200 °C/min and stayed at 200 °C for 4.2 min. The split vent was then opened again and the injector cooled down. Electronic flow control was used to maintain a constant helium carrier gas flow rate (1.8 ml/min). The GC oven temperature was held for 7 min at 40 °C, then increased by 6 °C/min to 260 °C and held at this temperature for 1 min. The mass spectra were taken at 70 eV with a scanning speed of 1 scan/s from m/z 30 to 350.

Processing of the data was performed by the help of the Saturn Software package 5.2.1. Tentative identification of floral scent components of the GC–MS spectra was carried out using the mass spectral data bases NIST 08, Wiley 8, MassFinder 3, and Adams (2007).

Scent samples were used to determine the compounds emitted from flowers or flower parts, and to determine the total amount of scent as well as the contribution of the single compounds to the total scent (percentage amount). To determine the total amount of scent, known amounts of monoterpenoids, benzenoids, and fatty acid derivatives were injected, and the mean peak area of these compounds was used for quantification.

2.5. Statistical analysis

To test whether the total amount of scent emitted differs during daytime, and among different flower parts, data were analysed using Repeated Measures ANOVAs (StatSoft, Inc., 2008). For graphical display of the temporal variation in scent during daytime (9 am to 6 pm), the total amount of scent was calculated in relation to the maximum amount of scent emitted by a specific flower. This standardisation was necessary as the total amount of scent emitted varied among flowers (Table 1).

Table 1
Total amount of scent and percentage amounts of the compounds emitted by six flowers (A–F) of two different plant individuals of *Ceropegia dolichophylla* at 9 am. KRI = Kovats retention index; tr: the amount was less than 0.05%. Values of more than 5.0% are printed in bold.

		Plant 1				Plant 2	
	KRI	A	B	C	D	E	F
Total amount trapped per min (ng)		56.9	19.1	46.1	10.1	16.0	28.6
N-bearing compounds							
N-3-Methylbutylacetamide	1141	2.2	tr	0.3	2.7	0.2	1.4
Spiroacetals							
m/z: 112,115,69,114,97,43	1152	**10.0**	**7.3**	**11.4**	2.6	**5.8**	**15.5**
m/z: 115,112,97,69,55,125	1319	**41.4**	**18.6**	**47.5**	**36.7**	**30.7**	**26.0**
m/z: 83,129,55,126,111,84	1331	**11.9**	**6.8**	**15.7**	**9.3**	**8.0**	**7.5**
Further unknown spiroacetals [a]		3.0^{9}	1.6^{9}	4.1^{9}	2.9^{9}	2.1^{7}	4.2^{9}
Aliphatics							
a Tridecene	1288	0.6	1.0	0.3	1.7	2.6	3.0
a Tridecene	1292	0.6	1.1	0.8	0.9	1.8	1.5
Tridecane	1300	**14.9**	**26.9**	**12.5**	**24.5**	**25.5**	**19.1**
a Pentadecadiene	1479	4.1	**10.1**	1.3	3.1	3.5	**7.4**
a Pentadecene	1483	**8.5**	**21.6**	3.2	**9.2**	**13.5**	**11.3**
a Pentadecene	1488	tr	tr	tr	0.1	0.5	0.1
Pentadecane	1500	0.3	1.1	0.4	0.9	0.9	0.7
2-Acethoxytridecane	1715	1.6	2.4	0.8	4.9	3.2	0.9
Irregular terpenes							
α-Ionone	1444	tr	0.1	0.2	0.1	tr	0.2
Unknowns [a]		0.7^{2}	1.3^{2}	1.5^{2}	0.6^{2}	1.6^{2}	1.1^{2}

[a] Unknown spiroacetals with a percentage amount of less than 1.0% and other unknowns were pooled with the superscript digit giving the number of pooled compounds.

2.6. Bioassay

To test whether flies can be attracted by the floral scent, the acetone scent sample (see above), representing the scent emitted during 7 h from three flowers (c. one fourth of the pooled sample), was used.

The bioassays were conducted in the field (Ecological-Botanical Garden of the University of Bayreuth). The acetone scent sample was offered in a small glass vial tucked into the soil and tested against a glass vial containing a similar amount of acetone only. The distance between the two vials was 30 cm. Bioassays took place twice (2 pm and 3 pm) on one day (September 2009; temperature: 24 °C, weather condition: full sun) lasting 40 min each. The position of scent sample and control was exchanged after 20 min each. Every fly approaching the vials within a range of 5 cm was caught (when sitting) using Eppendorf® tubes (1.5 ml).

3. Results

3.1. Flower scent

The floral scent of *Ceropegia dolichophylla*, as detectable by the human nose, can be described as sour-sweet with musky and sourish-metallic components.

The amount of floral scent emitted strongly differed among various flower parts (Fig. 2). The highest amount of scent was emitted by the very tip of the flower (lobe tips). The amount of scent emitted by the lower parts of the lobes, the lobe bases, was reduced to one sixth related to the very tip. The tube and the inflation emitted only trace amounts of scent.

During the period of measurement the total amount of scent seemed to depend on daytime, however, variation among individual flowers was high, and overall no significant differences in the scent emitted among different times were found (Fig. 3).

The total amount of scent trapped varied among flowers and was between 10 and 60 ng/min (Table 1). The flowers emitted one nitrogen bearing compound (N-3-methylbutylacetamide), spiroacetals, aliphatics, one irregular terpene (α-ionone), and a few compounds of unknown class. Spiroacetals were identified using their molecular ion combined with the characteristic pair of pronounced peaks built by retro-cleavage of the ring system

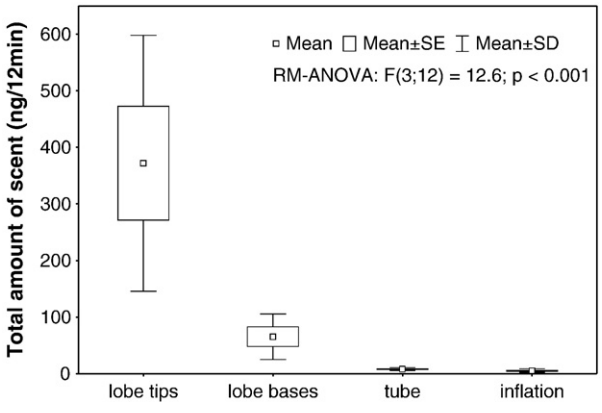

Fig. 2. Total amount of scent emitted by different floral parts of *Ceropegia dolichophylla* (five flowers from two plant individuals were used).

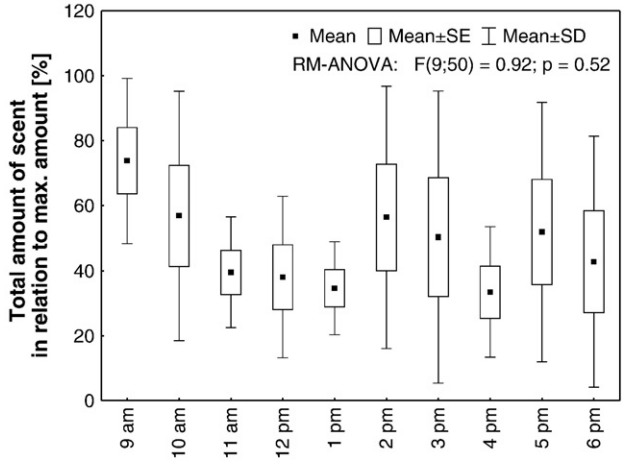

Fig. 3. Temporal pattern of floral scent emission in *Ceropegia dolichophylla*.

(see Francke and Kitching, 2001). Qualitative variation in scent was low, and most of the compounds were found in the samples of all flowers studied. Spiroacetals and aliphatics were the most abundant compound classes in all flowers. Spiroacetals contributed 34% to 79% to the total amount of scent emitted. Tridecane, one pentadecadiene, and one pentadecene were the most abundant aliphatics contributing 17% to 59% to the total amount of scent.

3.2. Flower visitors

Five insects (all flies) were found in 100 flowers collected in the native range of *C. dolichophylla* in China. These comprised a female *Desmometopa m-nigrum* (Milichiidae), two female *Neophyllomyza* sp. (Milichiidae), an unidentified species belonging to the Sciaridae and an individual insect in such bad condition that further identification was impossible (Table 2).

The 100 flies collected from flowers of *C. dolichophylla* in the greenhouse in Bayreuth, Germany, all belonged to *Desmometopa* (Milichiidae). Of these, 23 were sexed and identified to species level. All were females of *D. sordida*, and six thereof carried pollinaria of *C. dolichophylla* (Fig. 1C). Behavioural observations revealed that flies approached the flowers in the greenhouse in a zigzag manner, and landed mostly on the lobe tips. After landing many flies crawled around on the lobes, extended their proboscis and probed the lobe tips (Fig. 1B).

3.3. Bioassay

A flower scent sample (in acetone) of *C. dolichophylla* attracted 15 flies in one bioassay, and in a second biossay 12 flies during 40 min of observation each. No fly individual was attracted by the acetone controls. The first individuals approached within the first min after opening the extract tubes. All flies approached the tubes in a zigzag manner, against the direction of wind. All attracted flies were Milichiidae, and with the exception of one, all were *D. sordida*. One individual was a *Neophyllomyza acyglossa* female (Table 2).

4. Discussion

This study is the first in which scent emitted from *Ceropegia* flowers was analysed using dynamic headspace and GC-MS methods. The results show that scent in *C. dolichophylla* is mainly emitted from the corolla tips and from the morning until evening. The floral scent consisted mainly of spiroacetals and aliphatic compounds. The milichiid flies visiting the flowers in the native range in China and in a greenhouse in Germany are closely related. Bioassays with floral scent performed in the non-native range effectively attracted flies suggesting the importance of floral scent as pollinator attractant in *C. dolichophylla*.

Investigations of *C. dolichophylla* flowers collected in the native habitat revealed that female milichiliid flies (*Desmometopa m-nigrum* and *Neophyllomyza* sp.) and an unknown sciarid species are flower visitors and therefore potential pollinators. Species of both fly families and even of the genera *Desmometopa* and *Neophyllomyza* are already known visitors and potential pollinators for several *Ceropegia* species, but were not known as visitors of *C. dolichophylla* (Endress, 1996; Knuth, 1898-1905; Masinde, 2004; Vogel, 1961, 1993). The plants of *C. dolichophylla* cultivated in our greenhouse in Bayreuth, although far away from their native habitat, are regularly visited by females of *D. sordida* (Table 2). Furthermore, some of these flies also carried pollinaria clipped to their mouth parts, which suggests that they successfully act as pollinators of *C. dolichophylla* in the greenhouse (Fig. 1C). Indeed, the plants regularly set fruit, most likely as a result of geitonogamy or xenogamy (Meve, unpubl. data). The flies

Table 2
Number of dipterans found in *Ceropegia dolichophylla* flowers collected from plants in the native range (China) or from plants grown in Bayreuth, and number of dipterans attracted to floral scent in two bioassays. nd = not determined.

Family	Genus	Species	Sex	China	Bayreuth	Bioassays (Bayreuth)
Milichiidae	*Desmometopa* LOEW 1866	sp.	nd		76	
Milichiidae	*Desmometopa*	*m-nigrum* (ZETTERSTEDT 1848)	♀	1		
Milichiidae	*Desmometopa*	*sordida* (FALLÉN 1820)	♀		23	26[a]
Milichiidae	*Neophyllomyza*	*acyglossa* (VILLENEUVE 1920)	♀			1
Milichiidae	*Neophyllomyza* MELANDER 1913	sp.	♀	2		
Sciaridae			nd	1		
Unknown			nd	1		

[a] 15 and 11 flies, respectively were attracted in the two bioassays.

collected in the native range did not carry pollinaria, and we therefore do not know whether they act as pollinators. However, the *ex situ* pollinator *Desmometopa* has also been found in flowers collected in the native range and is most likely an *in situ* pollinator, too.

Sciaridae and Milichiidae both have worldwide distributions. The milichiid genus *Desmometopa* consists of 55 species, and the small black flies can easily be identified by an "M" on their frons. The two very similar species *D. m-nigrum* and *D. sordida* occurring as flower visitors in the greenhouse and natural habitat, respectively, are both cosmopolitan (Sabrosky, 1983). The milichiid fly genus *Neophyllomyza* consists of nine species distributed in all biogeographic regions (Brake, 2000, 2010). The family Sciaridae comprises 1700 described species. Milichiidae and Sciaridae are suggested to be saprophagous or phytophagous (only Sciaridae) food specialists (Vogel, 1961) or otherwise depend on carrion, fungal substrates, rotting plant or decaying organic material during the larval stages (Daly et al., 1998; Ollerton et al., 2009). Milichiid flies also have the noteworthy trait of kleptoparasitism — stealing food from other animals. They are known to feed on the prey (haemolymph or other secretions) of predatory arthropods, e.g. spiders (Eisner et al., 1991; Robinson and Robinson, 1977; Sabrosky, 1983; Sivinski, 1985; Sivinski and Stowe, 1980; Sivinski et al., 1999). Interestingly, with a few exceptions, only females are found to exploit such prey items (Sivinski, 1985; Sivinski et al., 1999). Volatile organic compounds from prey defense secretions, such as (E)-2-hexanol, hexyl butyrate, (E)-2-hexenyl butyrate, 2,4-hexadienyl hexanoate, and 2,4-hexadienyl butyrate are known to be responsible for the attraction of kleptoparasitic flies, including species of *Desmometopa* and *Neophyllomyza* (Aldrich and Barros, 1995; Beavers et al., 1972; Eisner et al., 1991; Sivinski et al., 1999; Zhang and Aldrich, 2004). Large amounts of glandular secretions are released from dead and injured insects, or from insects devoured by a predator (Zhang and Aldrich, 2004).

Volatile organic compounds, and specifically floral scents, are also suggested to be the main mode of attraction of fly pollinators in *Ceropegia* (Ollerton et al., 2009; Vogel, 1961). Our bioassay demonstrated that floral scent of *C. dolichophylla* alone is capable of attracting the fly pollinator *D. sordida*, as well as *N. acyglossa*, in the non-native range. We did not have the opportunity to test the attractiveness of the scent on the native pollinators. However, we assume that the identified potential milichiid pollinators are also attracted by the scent of the flowers in the native range, since all *Desmometopa* and all *Neophyllomyza* species have a very similar biology.

Our scent analyses demonstrate in a quantitative manner for the first time that the distal part of the flower (lobe tips) is mostly responsible for scent emission in a *Ceropegia* species, whereas other flower parts emit only very small amounts of these compounds (Fig. 2), and no other compounds (A. Heiduk, unpubl. data). This finding is consistent with the observations of Vogel (1961). He found in *Ceropegia* species other than *C. dolichophylla* that flower scent is produced by special epithelia ("osmophores") at the very tip of the flower and sniffing experiments allowed him to conclude that this flower part is also responsible for sent emission.

The flowers of *C. dolichophylla* open in the morning (between 4 and 5 am, in July) shortly before sunrise and start to wither and turn upside-down in the evening of the same day (around 8 pm) at sunset (A. Heiduk, unpubl. data). We measured scent emission from 9 am to 6 pm and results reveal that scent is continuously emitted during that time (Fig. 3). The presence of flies in some flowers already at 9 am (such flowers were not used for determining scent rhythmicity) and the occurrence of landings on flowers in the evening before sunset point towards an emission of floral scent throughout the whole time of anthesis.

C. dolichophylla flowers did not emit compounds which are known attractants for kleptoparasitic *Desmometopa* and *Neophyllomyza* (see above) or Sciaridae. Instead, flowers emit mainly three unknown spiroacetals, tridecane, a pentadecene, and a pentadecadiene (Table 1). Spiroacetals are unusual floral scent compounds with only six described so far (Knudsen et al., 2006): (E)-/(Z)-chalcogran (in few Orchidaceae, a Rubiaceae, and a Solanaceae species), (E)-/(Z)-conophthorin (in 13 families), 8,8-dimethyl-4-methylene-1-oxospiro[2.5]oct-5-ene (in *Osmanthus fragrans* Lour., Oleaceae), and spiro[4.5]dec-1-ene (in *Hedychium coronarium* König, Zingiberaceae). These six spiroacetals typically occur only in minor amounts in the scents, but (E)-conophthorin was an abundant compound in the scent of *Chelyocarpus ulei* Dammer (Arecaceae; pollinators unknown; Knudsen et al., 2001) and *Dorstenia turnerifolia* Fisch. & C. A. Mey (Moraceae; pollinators unknown; Kaiser, 2000). Tridecane is a widespread floral scent compound, while pentadecenes and pentadecadienes are not that widespread, and typically are only minor compounds in floral scents. It is unknown whether these spiroacetals and aliphatic compounds play a role in the communication between plants and pollinators. N-3-Methylbutylacetamide and 2-acetoxytridecane, with relative abundance of up to 3% and 5% in *C. dolichophylla* scent, respectively, were not described in floral scents before. 2-Acetoxytridecane, however, is already known as a secondary metabolite in plants, and occurs in trace amounts in the essential oil of leaves of members of the Rutaceae (Ivanova et al., 2004).

Interestingly, the spiroacetals, N-3-methylbutylacetamide, and 2-acetoxytridecane are all well known insect pheromones or occur at least in glandular secretions of insects. Spiroacetals occur e.g. in beetles, wasps, bees, ants, bugs, and fruit flies, and several of them have pheromonal functions (Francke and Kitching, 2001). N-3-Methylbutylacetamide occurs as an alarm pheromone in cockroaches (Farine et al., 2002) and wasps (Keeling et al., 2004), as a male sex pheromone in fruit flies (e.g. Wee and Tan, 2005), and was found in prothoracic glandular secretions of lacewings (Aldrich et al., 2009; Zhang et al., 2006). 2-Acetoxytridecane is a female sex pheromone in midges (Hillbur et al., 2000). Therefore, these compounds are widespread among insects, and insects of several orders, including Diptera, have olfactory capabilities to detect these compounds. We assume that kleptoparasitic *Desmometopa* and *Neophyllomyza* flies perceive these compounds, and that they play a role in finding appropriate feeding sites. *Desmometopa* flies, including *D. sordida* and *D. m-nigrum* are frequently recorded to be attracted to arthropods preying on honey bees

(Landau and Gaylor, 1987; Lopez, 1984; Sabrosky, 1983), and it would be possible that one of the compounds attracting these flies to *Ceropegia* flowers is present in the alarm pheromone of honey bees. However, the compounds emitted from *C. dolichophylla* are not known from honey bees though they might be present in other prey items attractive to *D. sordida* and *D. m-nigrum*. However, no data exists about the prey of these flies in the native range of *C. dolichophylla*.

Fly-pollinated trap flowers comparable to *Ceropegia* also occur in *Aristolochia* (Aristolochiaceae) and *Arisaema* (Araceae) species, and plants of these genera also attract their flies by specific scents (Barriault et al., 2010; Sakai, 2002). Scents of these plants can be described as faint earthy, meaty (resembling carrion), and mushroom like (see also Trujillo and Sérsic, 2006; Johnson and Jürgens, 2010). In contrast to *Ceropegia*, however, these plants are suggested to mimic brood sites of the visiting flies (Proctor et al., 1996).

Vogel (1961) suggested that the scent of *Ceropegia* could be an imitation of either food sites, breeding sites or sexual pheromones of flies, and our study supports the idea that *Ceropegia* (at least *C. dolichophylla*) mimics food sites, i.e. dead insects, of the pollinating flies. Our observations and those of Vogel (1961) indicating that flies scan the plant surface with their proboscides after landing, most likely in search for food, also support this hypothesis. In contrast, our data do not support the hypothesis that *Ceropegia* flowers imitate breeding sites (dung or rotting plant material; see above), as compounds found in the present study are not known from the flies´ breeding substrates or from plants mimicking such substrates (e.g. dung, see Johnson and Jürgens, 2010; Jürgens et al., 2006). The hypothesis that the flowers mimic male sex pheromones cannot yet be evaluated as the sexual pheromones of these flies are unknown. In order to further understand the myiophilous *C. dolichophylla* pollination system, it is necessary to identify the unknown spiroacetals and aliphatic compounds, and to determine their attractiveness to fly pollinators in the natural habitat.

Acknowledgements

This article is dedicated to S. Vogel, who pioneered the work on pollination in *Ceropegia* including the description of the osmophores. We thank Y. Zhou and H. Kong for providing us with seeds and flowers of *Ceropegia dolichophylla*, and J. Aldrich for discussions about the chemistry of defense secretions in lacewings. The comments of anonymous referees on a previous version of the manuscript were very helpful.

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Edited by AM Viljoen

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