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FLORAL SCENT AND POLLINATION ECOLOGY OF ORCHIDS FROM THE ATLANTIC FOREST OF SOUTHEASTERN BRAZIL

FRAGRÂNCIAS FLORAIS E A ECOLOGIA DA POLINIZAÇÃO DE ORQUÍDEAS DA MATA ATLÂNTICA DO SUDESTE DO BRASIL

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Thesis presented to the Institute of Biology of the State University of Campinas in partial fulfillment of the requirements for the degree of Doctor in Plant Biology

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A vida é uma caminhada e

subindo e descendo as escadas

destas montanhas molhadas

"Cipó Caboclo tá subindo na Virola
Chegou a hora do Pinheiro balançar
Sentir o cheiro do mato, da Imburana
Descansar, morrer de sono na sombra da Barriguda
De nada vale tanto esforço do meu canto
Pra nosso espanto tanta mata haja vão matar
Tal Mata Atlântica e a próxima Amazônica
Arvoredos seculares impossível replantar
Que triste sina teve o Cedro, nosso primo
Desde de menino que eu nem gosto de falar
Depois de tanto sofrimento seu destino
Virou tamborete, mesa, cadeira, balcão de bar
Quem por acaso ouviu falar da Sucupira
Parece até mentira que o Jacarandá
Antes de virar poltrona, porta, armário
Mora no dicionário, vida eterna, milenar"

(Jatobá)

ABSTRACT

Floral volatile organic compounds (VOCs) play important roles in plant-pollinator interactions. Floral biology, pollination and breeding systems as well as floral VOCs of orchid species from the Atlantic forest were investigated to understand plant-pollinator communication and its importance to plant reproduction. The floral biology, pollination and breeding systems of *Catasetum cernuum*, *Dichaea pendula*, *D. cogniauxiana*, *Gongora bufonia*, *Pabstia jugosa, Promenaea xanthina*, *Warrea warreana*, *Zygopetalum crinitum*, *Z. maculatum* and *Z. maxillare* were studied *in situ* in southeastern Brazil. Floral scent was assessed using techniques of headspace collection and GC-MS. We analysed the matrix of 'individual × floral VOCs' using multivariate techniques (UPGMC, PCA, ICA, MRPP and ANOSIM) in order to classify the species according their floral VOCs, determining distinctive compounds associated both to species and their pollinators, and look for significant differences among species. *Catasetum cernuum, D. pendula* and *Gongora bufonia* offered perfumes for their pollinators – male euglossine bees. Noteworthy, *D. cogniauxiana* offered developing ovules to pollinators, weevils *Montella* sp. nova which actively self-pollinate flowers before laying eggs on the stigmatic cavity, what constitute a novel nursery pollination system. The remaining species offered no floral resources. Only female carpenter bees pollinated *Z. crinitum*. Bumble-bees pollinated *W. warreana, Z. maculatum* and *Z. maxillare*. The latter was also pollinated by *Centris confusa. Warrea warreana* and *Z. crinitum* were visited but not pollinated by male orchid-bees. Breeding system varied from complete self-incompatibility (*D. pendula*) to self-compatibility with spontaneous selfing (*D. cogniauxiana* and *Z. maxillare*). Most VOCs were common to other floral scents. However, flowers of *C. cernuum* and *G. bufonia* emitted the unusual *(e)*-epoxyocimene and *D. pendula* emitted the unusual 2-methoxy-4-vinylphenol. The similarity between the floral scents of *C. cernuum* and *G. bufonia* could be associated to the exclusive pollination by male *Eufriesea violacea* bees, likely characterizing an example of pollinator-mediated evolutionary convergence of floral chemistry. *Zygopetalum crinitum* presented an exclusive blend of VOCs, mainly benzenoids. According to floral scent composition, *Pabstia jugosa*, *Pro. xanthina* and the *Zygopetalum* spp. composed a separate consistent group.

Although we did not record pollinators in *Pab. jugosa* and *Pro. xanthina*, our analyses indicated these species are deceit-pollinated by Apidae. There is a close phylogenetic relationship between deceit-pollinated and euglossine-pollinated orchid species. Additionally, a private channel with a distinctive floral VOC bouquet may determine specialization of *C. cernuum* and *G. bufonia* in male *Euf. violacea* as well as the specialization of *Z. crinitum* in carpenter bees within flowers pollinated by Apidae.

Keywords: Apidae, Atlantic forest, bee, chemical ecology, deceit-pollination, *Dichaea*, Orchidaceae, *Promenaea*, sensorial exploitation, *Warrea*, *Xylocopa*, *Zygopetalum*.

RESUMO

Compostos orgânicos voláteis (COVs) emitidos pelas flores têm um papel importante na mediação das interações planta-polinizador. Neste trabalho, foram estudados a biologia floral, a polinização, o sistema reprodutivo e a composição química dos COVs florais de 10 espécies de orquídeas nativas da Mata Atlântica do Sudeste do Brasil: *Catasetum cernuum*, *Dichaea pendula*, *D. cogniauxiana*, *Gongora bufonia*, *Pabstia jugosa, Promenaea xanthina*, *Warrea warreana*, *Zygopetalum crinitum*, *Z. maculatum* e *Z. maxillare.* As fragrâncias florais foram coletadas usando técnicas de coleta do *headspace* floral e sua composição química determinada por cromatografia gasosa acoplada a espectrometria de massas (GC-EM). A matriz de dados 'indivíduos × COVs florais' foi analisada com técnicas de análise multivariada (UPGMC, PCA, ICA, MRPP e ANOSIM) a fim de classificar essas espécies de acordo com seus COVs florais, determinando os voláteis distintivos associados a espécies e polinizadores, e buscar por diferenças significativas entre as espécies. *Catasetum cernuum, D. pendula* e *Gongora bufonia* oferecem perfumes como recurso floral a seus polinizadores – machos de abelhas Euglossina. Notoriamente, *D. cogniauxiana* oferece seus óvulos em desenvolvimento às larvas de seus polinizadores, gorgulhos *Montella* sp. nova, que autopolinizam ativamente as flores antes de depositar seus ovos nos estigmas, caracterizando um novo caso de polinização de berçário. As demais espécies não ofereceram recursos florais aos polinizadores. *Zygopetalum crinitum* foi polinizado exclusivamente por fêmeas de mamangavas *Xylocopa* sp. Abelhas *Bombus* sp. polinizaram *W. warreana, Z. maculatum* e *Z. maxillare*, sendo o último também polinizado pela abelha solitária *Centris confusa*. *Warrea warreana* and *Z. crinitum* foram visitados por machos de abelhas Euglossina que, no entanto, não as polinizavam. O sistema reprodutivo variou da completa autoincompatibilidade (*D. pendula*) à autocompatibilidade com polinização espontânea (*D. cogniauxiana* and *Z. maxillare*). A maioria dos COVs é comum à outras fragrâncias florais. Entretanto, as flores de *C. cernuum* e *G. bufonia* emitiram o pouco comum (*e*)-epoxyocimene e *D. pendula* emitiu o raro 2-methoxy-4-vinylphenol. A similaridade entre as fragrâncias de *C. cernuum* e *G. bufonia* estão associadas à polinização exclusiva por machos da abelha

Eufriesea violacea, caracterizando provavelmente um exemplo convergência evolutiva da fragrância floral mediada pela seleção por um único polinizador. *Zygopetalum crinitum* apresentou um buquê exclusivo de COVs derivados do ácido shiquímico. De acordo com a composição da fragrância floral, *Pab. jugosa, Pro. xanthina* e as espécies de *Zygopetalum* compuseram um grupo a parte. Apesar de os polinizadores de *Pab. jugosa* e *Pro. xanthina* não terem sido registrados, as análises sugerem que essas espécies seriam polinizadas por engodo por abelhas. Assim, foi constatada uma relação filogenética próxima entre orquídeas polinizadas por engodo e orquídeas polinizadas por machos Euglossina. Ademais, canais exclusivos de comunicação química determinam a especialização de *C. cernuum* e *G. bufonia* em machos de *Euf. violacea* e de *Z. crinitum* em *Xylocopa* dentre outras espécies polinizadas por Apidae.

Palavras-chave: abelha, Apidae, Floresta Atlântica, ecologia química, engodo, exploração sensorial, *Dichaea*, Orchidaceae, *Promenaea*, polinização, *Warrea*, *Xylocopa*, *Zygopetalum.*

SUMMARY

INTRODUÇÃO GERAL

Plantas se comunicam quimicamente com indivíduos da mesma espécie bem como com os predadores de seus herbívoros (Dicke and Baldwin 2010). Além disso, os grupos de animais (em sua maioria insetos) que polinizam a maior parte das espécies de orquídeas se orientam por diferentes canais de comunicação para encontrar as flores e os recursos associados a estas. Assim, sinais visuais (físicos) e sinais químicos são utilizados por esses animais para reconhecer fontes de recursos durante sua busca por alimento, materiais e locais para nidificação e feromônios (Wright and Schiestl 2009; Schiestl and Schlüter 2009). Assim, os ompostos orgânicos voláteis (COVs) apresentam papel fundamental na comunicação entre plantas e outros organismos, especialmente, na interação entre plantas e seus polinizadores e dispersores (Knudsen et al. 2006; Hossaert-McKey et al. 2010; Midgley et al. 2015).

A onipresença e a complexidade da comunicação química no reino vegetal levaram pesquisadores a propor a existência de uma linguagem entre as plantas e outros organismos que até então eram considerados incapazes de se comunicar e trocar informações de maneira semelhante ao que ocorre entre seres humanos e outros animais (Pineda et al. 2010; Dicke and Baldwin 2010). Assim, alguns pesquisadores propõem que uma abordagem comparável à de estudos linguísticos seja utilizada em estudos da semioquímicos envolvendo plantas para se avançar com o conhecimento e tecnologias (Gagliano and Grimonprez 2015).

Os COVs compõem as fragrâncias florais e estas, em geral, são caracterizadas por um buquê complexo e específico de cada espécie vegetal, formado por uma mistura de compostos orgânicos voláteis produzidos e emitidos pela flor diretamente na atmosfera (Raguso 2008; Gong et al. 2014). Os COVs florais funcionam como atrativos florais e atuam no reconhecimento dos recursos florais pelos polinizadores e sua produção e emissão podem estar sujeitas à seleção mediada por polinizadores ao longo da história evolutiva das plantas (Williams and Whitten 1983; Gerlach and Schill 1991; Gang 2005;

Schäffler et al. 2015). Assim, a maioria dos estudos de caracterização dos compostos do *headspace* em angiospermas procura uma relação destes compostos com os polinizadores e os sistemas de polinização (Knudsen and Mori 1996; Raguso et al. 2003; Dötterl et al. 2005; Majetic et al. 2009; Schiestl and Dötterl 2012; Oelschl et al. 2014; Schäffler et al. 2015), sendo que orquídeas constituem o grupo de plantas com o maior conhecimento acumulado sobre voláteis florais até o momento (Kaiser 1993; Knudsen et al. 2006).

A família Orchidaceae é uma das mais diversificadas entre as angiospermas, com cerca de 24500 espécies espalhadas por todos os continentes exceto a Antártica, sendo que 1257 espécies, ou 5%, ocorrem no bioma Mata Atlântica (Dressler 2005; Stehman et al. 2009). Esses números colocam a família Orchidaceae como a maior entre as monocotiledôneas e um importante componente da flora desse bioma, compondo 8% das espécies de plantas vasculares (Stehman et al. 2009).

Quanto aos recursos florais, as orquídeas podem ser primariamente classificadas entre espécies que oferecem recurso ao polinizador e espécies que são polinizadas por engano, não oferecendo recurso algum (Cozzolino and Widmer 2005). Exemplos de recursos florais oferecidos por orquídeas aos seus polinizadores são: néctar, perfumes (van der Pijl and Dodson 1969), pseudo-pólen (Pansarin and Amaral 2006), óleos (Steiner 1998) e, raramente pólen (Tremblay et al. 2005). Assim como nas Angiospermas em geral, a maioria das espécies de Orchidaceae apresenta melitofilia (polinização por abelhas) como sistema de polinização (van der Pijl and Dodson 1969), sendo que a miiofilia (polinização por moscas) também pode compor um dos mais importantes sistemas de polinização na família (Borba 2001; Paudel et al. 2015).

Neste sentido, é notório que em diversas espécies de orquídeas, a fragrância apresenta papel fundamental na atração dos polinizadores. Dependendo da espécie em questão o perfume pode funcionar como um atrativo para flores que oferecem néctar ou como um engodo, simulando o aroma de uma flor com recurso ou de fêmeas de determinadas espécies de abelhas e vespas (Alcock 2005; Schiestl 2005; Raguso 2008). Em subtribos como Catasetinae e Stanhopeinae (Epidendroideae: Cymbideae *sensu* Pridgeon et al., 2006), por exemplo, o odor pode até ser oferecido como o próprio recurso a ser coletado por abelhas da tribo Euglossina, os conhecidos polinizadores destas plantas (Vogel 1963; Dressler 1968; Roubik and Ackerman 1987; Eltz et al. 2005, 2006). Em plantas desses grupos, a composição do odor é um fator determinante e intimamente relacionado à reprodução, podendo ser usado como caráter na sistemática das espécies (Gerlach and Schill 1991).

A literatura sobre Orchidaceae vem crescendo nas últimas décadas, com aumento evidente de conhecimentos sobre sua reprodução, genética e sistemática (Chase et al. 2009; Fay and Chase 2009; Givnish et al. 2015). Apesar de ser de longe a família com o maior número de espécies com a fragrância floral conhecida (417 espécies distribuídas em 104 gêneros, *sensu* Knudsen et al., 2006), a maioria dos trabalhos que caracteriza a fragrância floral de orquídeas não apresenta dados empíricos sobre os visitantes florais e polinizadores, não permitindo o estabelecimento de uma relação direta entre determinados grupos de polinizadores e a composição química das fragrâncias destas plantas. Ademais, pouco é conhecido sobre as interações da maioria das espécies com seus polinizadores, sendo os estudos de campo importantes para confirmar as especulações feitas a partir de caracteres morfológicos desde o século XIX (Darwin 1877; Micheneau et al. 2009; Bronstein et al. 2014).

Tendo em vista a importância desse grupo de plantas para conservação e manutenção da biodiversidade de ecossistemas, bem como seu valor ornamental e comercial, é necessário destacar que espécies de orquídeas estão entre as plantas mais ameaçadas pela destruição do habitat e coleta ilegal (Benzing 1998; Flores-Palacios and Valencia-Diaz 2007; Swarts et al. 2009), a despeito de seu potencial de uso como espécies “bandeira” para a preservação de ecossistemas, uma vez que concentram grande número de admiradores e estudiosos. Portanto, são essenciais estudos envolvendo observações diretas no campo para elucidar as interações com os polinizadores e seu comportamento e os mecanismos relacionados à polinização, associados à caracterização dos compostos voláteis e a reprodução das espécies da Mata Atlântica.

Este trabalho teve como objetivo geral determinar os COVs das fragrâncias florais durante os estudos de ecologia da polinização de espécies selecionadas de orquídeas da Mata Atlântica e relacionar estes dados aos polinizadores e seu comportamento. Especificamente, ao longo dos quatro capítulos que se seguem, as questões abordadas foram as seguintes:

- Qual a composição específica dos COVs das fragrâncias florais de cada espécie?
- Quais são visitantes florais e polinizadores, seu comportamento e os mecanismos de polinização?
- Qual o tipo de sistema reprodutivo?
- Que interações ecológicas são necessárias para a reprodução dessas plantas no habitat?
- Existem barreiras naturais aos cruzamentos interespecíficos entre espécies que florescem na mesma época? Quais seriam?
- É possível predizer os polinizadores a partir da análise multivariada dos componentes da fragrância floral das espécies estudadas?

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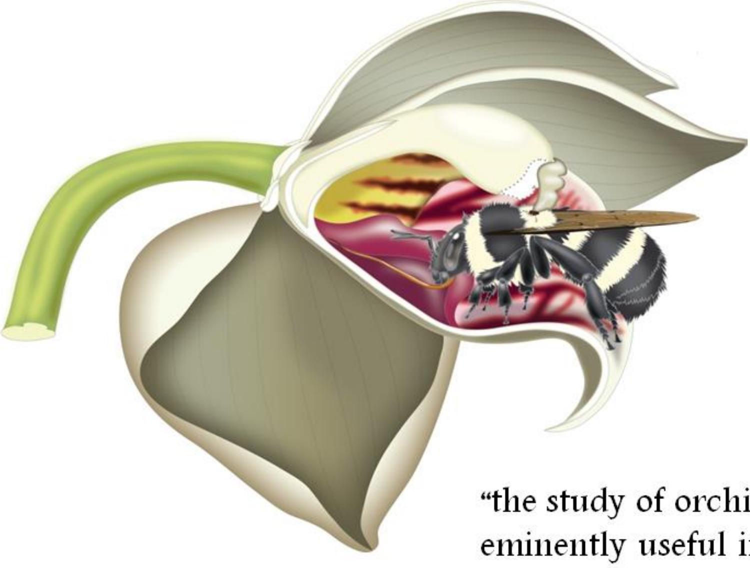
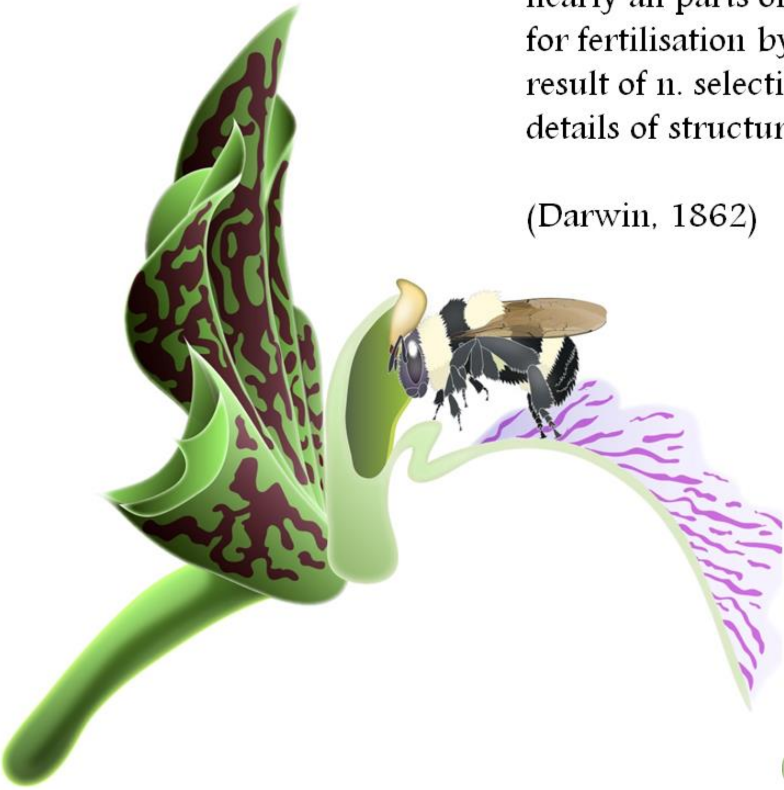


> “the study of orchids [has been found] eminently useful in showing me how nearly all parts of the flower are coadapted for fertilisation by insects, & therefore the result of n. selection,—even most trifling details of structure“
>
> (Darwin, 1862)



Chapter I

Floral scents and pollinators of Zygopetalinae orchids: diverse pollination systems in a group previously only known to be euglossine-pollinated

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Floral scents and pollinators of Zygopetalinae orchids: diverse pollination systems in a group previously only known to be euglossine-pollinated

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Summary

- *Background and aims* Floral volatile organic compounds (VOCs) play important roles in plant-pollinator interactions. We investigated floral biology, pollination and breeding systems, and floral VOCs of Zygopetalinae species to understand the relation between floral scent and attraction of specific pollinators.
- *Methods* We performed monthly censuses, focal observations and controlled pollinations in natural populations of *Dichaea pendula*, *D. cogniauxiana*, *Pabstia jugosa, Promenaea xanthina*, *Warrea warreana*, *Zygopetalum crinitum*, *Z. maculatum* and *Z. maxillare* from south-eastern Brazil. We collected and analysed floral scent using SPME/GC-MS. In order to group the species according the affinities of their floral VOCs and determine distinctive compounds associated both to species and their pollinators, we analysed the matrix of 'individual × floral VOCs' using multivariate techniques (UPGMC, PCA and ICA).
- *Key Results Dichaea pendula* and *D. cogniauxiana* offered respectively perfumes and developing ovules to pollinators, namely, male euglossine bees and weevils. The remaining species offered no floral resources. Only female carpenter bees pollinated *Z. crinitum*. Bumble-bees pollinated *W. warreana, Z. maculatum* and *Z. maxillare*. The latter was also pollinated by *Centris confusa. Warrea warreana* and *Z. crinitum* were visited but not pollinated by male orchid-bees. Breeding system varied from complete self-incompatibility (*D. pendula*) to self-compatibility with spontaneous selfing (*D. cogniauxiana* and *Z. maxillare*). Most VOCs were common to floral scents of other plant species; however, flowers of *D. pendula* emitted the unusual 2-methoxy-4-vinylphenol. *Zygopetalum crinitum* presented an exclusive blend of VOCs, mainly comprised of benzenoids. Blends emitted by *Pab. jugosa*, *Pro. xanthina* and the *Zygopetalum* spp. composed a separate consistent group.
- *Conclusion* Although we did not record pollinators in *Pab. jugosa* and *Pro. xanthina*, our analyses indicated these species are deceit-pollinated by Apidae. There is a close phylogenetic

relationship between deceit-pollinated and euglossine-pollinated orchid species. Additionally, a private channel with a distinctive floral VOC bouquet may determine specialization in carpenter bees within flowers pollinated by Apidae.

Keywords: Apidae, Atlantic forest, bee, chemical ecology, deceit-pollination, *Dichaea*, Orchidaceae, *Promenaea*, sensorial exploitation, *Warrea*, *Xylocopa*, *Zygopetalum*.

Introduction

Communication between plants and insect pollinators usually involves floral volatile organic compounds (VOCs) and the study of these chemicals has been important to understand the ecology and evolution of plant-pollinator interactions (Raguso et al. 2015; Schiestl 2015). Generally, floral scents consist of complex mixtures of VOCs specific to each plant species, in which each compound may vary in its presence/absence and relative proportion as well as in its behavioural function (Knudsen et al. 1993, 2006; Kessler et al. 2013). Thereby, floral VOCs can be used as a taxonomic character as well as to understand plant-animal interactions and even predict the pollinators (Baldwin et al. 1997; Oelschl et al. 2014; Gong et al. 2014). Together with visual cues, VOCs are important at both long-distance orientation and short-distance recognition of floral resources by pollinators (Williams and Whitten 1983; Wright and Schiestl 2009). Long-distance attraction by odour cues might be particularly important in the tropical forest environment, where most flowers on a plant assemblage are not readily visible to pollinators. Once floral scent signalling can enhance sexual reproduction in plants by mediating plant-pollinator interactions (Schiestl and Johnson 2013; Schiestl 2015), providing conspicuous odour pathways to pollinators may be an important adaptive strategy for epiphytic herbaceous tropical forest plants such as orchids.

The family Orchidaceae comprises huge morphofunctional and chemical floral diversity (Kaiser 1993; Gravendeel et al. 2004; Knudsen et al. 2006; Mondragón-Palomino and Theissen 2009), both which are related to the use of different groups of pollinators or even different parts of a pollinators body for transporting pollen between orchid individuals (van der Pijl and Dodson 1969; Roubik and Ackerman 1987; Schiestl and Schlüter 2009). For instance, morphological and/or chemical specializations of orchids may act as mechanisms to avoid the negative effects of competition for pollinators and interspecific pollen transfer in sympatric plants (Waser 1983; Galen and Gregory 1989; Mitchell et al. 2009). Though, these mechanisms may be substituted, reinforced or complemented by segregation of flowering phenology in time (Mitchell et al. 2009; Sun et al. 2011; van der Niet et al. 2011). Thus, orchids are an excellent model to study the relationships among plants and their pollinators mediated by morphological adaptations and floral volatiles (Bronstein et al. 2014). Moreover, *in situ* ecological studies are essential to understand the actors and roles that are involved in plant-animal interactions mediated by VOCs.

Despite the high diversity of Zygopetalinae orchids (*ca.* 35 genera and 420 species, Whitten et al. 2005; The Plant List 2010) and the high levels of endemism in the Brazilian Atlantic Forest (ca. 11.9% of the Zygopetalinae species are endemic to this biome, Barros et al. 2010), few empirical data is available for the pollination in this subtribe. For instance, literature provides punctual observations of flower-visiting and pollinaria-carrying by Euglossini bees in some *Dichaea* species (van der Pijl and Dodson 1969; Folsom 1985; Roubik and Ackerman 1987; Singer and Sazima 2004; G. Gerlach, personal communication) and species of *Chaubardia* Rchb.f., *Chaubardiella* Garay, *Chondroscaphe* (Dressler) Senghas & G.Gerlach, *Kefersteinia* Rchb.f., *Pescatoria* Rchb.f. and *Warrea warreana* (van der Pijl and Dodson 1969; G. Gerlach and H. Heider, personal communications). Thus, a more comprehensive approach about the pollination of this subtribe is needed.

In this work we studied the floral biology, pollination and reproductive biology, flowering phenology as well as floral VOCs of eight species of Zygopetalinae orchids from the genera *Dichaea, Pabstia*, *Promenaea*, *Warrea* and *Zygopetalum* in the Atlantic forest of south-eastern Brazil. Moreover, we tested whether chemical composition of the floral scent emitted by sympatric Zygopetalinae species is related to the visitation of pollinators. We then tested whether species sharing bee pollinators overlap or segregate in time possibly as a response to competition for pollinators. Finally, we discuss the isolating mechanisms of the pollinator-sharing species.

Material and methods

Study species and sites

We studied populations of *Dichaea cogniauxiana* Schltr., *D. pendula* (Aubl.) Cogn., *Pabstia jugosa* (Lindl.) Garay, *Promenaea xanthina* Lindl., *Warrea warreana* (Lodd. ex Lindl.) C.Schweinf., *Zygopetalum crinitum* Lodd., *Z. maculatum* (Kunth) Garay and *Z. maxillare* Lodd., which occur in sympatry in one of the study areas (Serra do Mar, SM). *Dichaea cogniauxiana, D. pendula, Pab. jugosa, Pro. xanthina* and *Zygopetalum crinitum* grow as epiphytes in several host trees (*Tibouchina pulchra* – Melastomataceae, *Peltophorum dubium* and *Inga* spp. - Fabaceae) or on tree fern species (*Cyathea* spp.). *Zygopetalum maxillare* grows exclusively on the tree ferns like *Dicksonia sellowiana*, *Cyathea* spp. and *Alsophila* spp. *Zygopetalum maculatum* is terrestrial or lithophytic and thrives in sun-irradiated habitats of forest borders nearby roads or in debris among rocky outcrops in the Atlantic Forest of south-eastern Brazil (Barros et al. 2014) (Fig. 1) .

Most of the fieldwork at SM study site was carried out between 2011 and 2014 in a montane Atlantic forest area of the Serra do Mar State Park (from 800 m to 1000 m a.s.l. in an ombrophilous dense forest, IBGE 2012) located in the Serra do Mar mountain range in São Paulo state (23°26'08"S - 45°13'22.5"W and 23°19'55"S - 45 º05'49"W). Additionally, we studied the reproductive biology and pollination of *Z. crinitum* and *Z. maxillare* in two other study areas. We also studied *Z. crinitum* at the natural reserve of Serra do Japi (SJ), within the boundaries of the municipality of Jundiaí (23°11'S, 46º52'W; 700 – 1300 m a.s.l.) from 2012 to 2014, and *Z. maxillare* at Itatiaia National Park (INP; 22º27'S; 44°36'W, 750-1,100 m a.s.l.) in 2007 and 2014. The latter two study areas are also located in south-eastern Brazil and characterized by montane and sub-montane ombrophilous dense forest (IBGE 2012).

For the floral biology, resources and volatile collection, three to nine individual plants of each species were collected in the field, transferred to the greenhouse in the University of

Campinas, and kept under cultivation until flowering. Voucher specimens were deposited in the herbarium of the University of Campinas (UEC; *D. cogniauxiana*: C.E.P. Nunes, 03 17-Feb-2011; *D. pendula*: E. Melo *et al.* 2555, 14-Nov-1998; R.B. Singer, 99/38 25-Nov-1999; C.E.P. Nunes, 5 19-Nov-2012; *Pab. jugosa*: C.E.P. Nunes, 13 28-Jul-2014; *Pro. xanthina*: R.B. Singer 200016 25-Jan-2000; *W. warreana*: C.E.P. Nunes 6 12-Jan-2013; *Z. crinitum*: C.E.P. Nunes 7 28-Jul-2015; *Z. maculatum*: C.E.P. Nunes 4 15-May-2015; *Z. maxillare*: R.G. Udulutsch *et al.* 674 23-Apr-2002).

Breeding system

The breeding system of all the species was assessed using controlled pollination experiments (Radford *et al.* 1974; Dafni *et al.* 2005). The pollination experiments included (i) hand cross-pollination (flowers were emasculated and hand-pollinated using one pollinarium from an individual at least 10 m away), (ii) hand self-pollination (flowers were hand-pollinated using each plant's own pollinarium), (iii) spontaneous self-pollination (unmanipulated flowers were bagged without further treatment), (iv) emasculation (flowers had their anthers removed, then flowers were bagged) and (v) natural pollination (flowers were left exposed to pollinators) (Table S1). For all the pollination treatments (manual self- and manual cross-pollination), a whole pollinarium was used in each experimental pollination event. For *Pab. jugosa* and *Pro. xanthina*, the number of individuals used in the controlled pollination experiments was severely reduced and not all the experiments could be performed due to the rareness of the species in the study site, where both species were represented by no more than 10 individuals (Table S1).

Floral biology, resources and pollinators

For the species in which the presence of floral resources could not be directly inferred in field, namely *Pab. jugosa*, *Pro. xanthina*, *W. warreana* and the three *Zygopetalum*, flowers from cultivated individuals were examined and dissected in order to identify the presence of any floral resource such as oil or nectar. The spurs formed by the sepals in *W. warreana* and by the lip in *Pab. jugosa* and *Zygopetalum* were checked for nectar in amounts higher than 4 µl with micro-syringes (n = 5 flowers from different individuals). In order to test for the presence of nectar, anthetic flowers with no more than three days old from greenhouse cultivated plants of *Pro. xanthina* and the *Zygopetalum spp.* (n = 3 flowers from at least two individuals) were histochemically tested for soluble reducing sugars, i.e. nectar, using Fehling's reaction (Fehling 1858; Díaz-Castelazo et al. 2005).

Overall, a total of 604 h of observation for floral visitors was performed. During each observation period, one to four individuals bearing 1-10 flowers each were observed *in situ* or recorded with a camcorder (DCR-SR68 Sony, Tokyo, Japan). For each species studied, 19-89 h (min-max) of observation was performed (Table S1). In the main study site, SM, a total of 480 h of observations of floral visitors was performed during the flowering seasons from 2010 to 2014, from 07:00 to 19:00 h, covering both diurnal and crepuscular periods. In SJ, the observations on *Z. crinitum* flowers were carried out in August 2012 and August 2013. Also to detect possible nocturnal pollination, only in SJ where logistical conditions allowed us, flowers (n = 20; four inflorescences; four plants) were tagged in the afternoon (2012 and 2013 flowering periods) and examined in the early morning around 08:00 h. In INP, observations of flower visitors of *Z. maxillare* were performed in February and March 2007 and 2014 with naked eye in open or partially covered days, counting 41 h (Table S1).

The time, sequence and duration of anthesis (i.e., the period in which the corolla was open and male or female functional organs were present) were assessed *in situ*. Additionally, the flower lifespan was assessed for bagged inflorescences (unavailable to herbivores) of at least two individuals of each studied *Zygopetalum* species. Floral visitors removing and depositing pollinaria or touching the viscidium and stigmatic region of flowers were considered to be pollinators. The behaviour of visitors and pollinators were analysed from digital videos and images from focal observations.

Volatile collection and analysis of floral scents (SPME and GC-MS)

Floral scent was collected in the greenhouse using solid phase micro-extraction (SPME), and analysed it in laboratory, using gas chromatography coupled to mass spectrometry (GC-MS) as described below. When the flowers opened, inflorescences or parts of them were wrapped in polyester bags (27 × 41 cm for *Zygopetalum* and *Warrea*; 13.5 × 20.5 for *Dichaea*, *Pabstia* and *Promenaea*) and left for one to three hours to concentrate floral VOCs and reach the flower-air equilibrium. Thereafter, bags were perforated with a pin and their VOC containing air was exposed to a solid phase micro-extraction (SPME) syringe carrying a polydimethylsiloxane fibre (PDMS, 100 µm, Supelco, Bellefonte, PA) for 15 min. Collections were performed on sunny days at 20-30 ºC, the same day-time as fragrances were most often detected by the human sense of smell under natural conditions in the field (from 10:00 to 13:00 h).

Immediately after collection, SPME fibre samples were directly injected into a gas chromatograph (2010A, Shimadzu, Tokyo, Japan) that was coupled to a quadrupole mass spectrometer (QP2010, Shimadzu) using a DB5 capillary column (30 m length, 0.32 mm internal diameter and 0.25 µm film thickness, J&W Scientific, Folsom, CA, USA) and helium as a carrier gas (flow of 1 mL.min^{-1}). Injection was performed in splitless mode, and the fibre was kept for 20 min in the injector at 200 ºC with the transfer line at 240 ºC to elute VOCs. The oven temperature started at 50 ºC and then increased by 10 ºC min^{-1} to a maximum temperature of 250 ºC and was then held for 10 min until the end of the run. Mass spectra were recorded by electron impact (EI) at 70 eV using the SIM mode. We calculated the Kovats Retention Index (RI) of each compound peak from a previously injected, homologous series of n-alkanes (C8-C20). Compound peaks were identified by comparison of the mass spectra and RIs to those of the NIST05 and NIST online library (Linstrom and Mallard 2011) and *The Pherobase* semiochemical database (El-Sayed 2012). In the case of 2-methoxy-4-vinylphenol, the mass spectra and retention time were compared with those of the analytical standard (Sigma-Aldrich, Saint Louis, IL, USA).

Floral scent statistical analysis

Because our 'individuals × VOCs' matrix of floral scents met the assumption of multivariate homogeneity of group dispersions (variances, ANOVA $F_{7,23} = 0.818$, $p = 0.585$, test performed with the VEGAN package [version 2.0–5; Oksanen et al. 2012] in R), but not the assumption of multivariate normality of variances (Shapiro–Wilk normality test, W = 0.23, p-value = $1.4\ e^{-15}$, test performed with the MVNORMTEST package [Jarek 2012] in R), a non-parametric tests was used to characterise the differences on floral scent among samples and Zygopetalinae species. Hellinger transformation, a relativisation by row (sample unit) totals followed by taking the square root of each element in the matrix, to make the floral scent data containing many zeros (e.g., compounds completely absent in certain species, but present in others) suitable for multivariate analysis (Legendre and Legendre 1998; Legendre and Gallagher 2001). A non-parametric multiple response permutation procedure (MRPP) with the average Bray–Curtis distances among samples weighted to group size and 999 random permutations (Mielke and Berry 2001; McCune et al. 2002) was first conducted to test the null hypothesis of no difference in floral scent (relative amounts, in %) among species. The MRPP test was performed with the VEGAN package (version 2.0–5;Oksanen et al. 2012) in R. An analysis of similarities (ANOSIM) using the average Bray–Curtis distances among samples and 1000 permutations with the VEGAN package (version 2.0–5; Oksanen et al. 2012) in R was also conducted as an alternative way to statistically test whether there is a significant difference in floral scent composition among Zygopetalinae species. To detect floral scent compounds whose presence is statistically associated with certain species, an indicator compound analysis (ICA) with 999 random permutations was performed. The computed indicator value of each compound reflects both its relative abundance (specificity – 'A', the probability that the odour compound belongs to the target species) and its relative frequency (fidelity – 'B', the probability of finding the odour compound in other species). The associated *p*-values determined whether specific compounds are significant indicators of certain species (Dufrêne and Legendre 1997; De Caceres and Legendre 2009). The ICA was performed with the INDICSPECIES package (De Caceres and Legendre 2009) in R.

To characterise floral scent dissimilarities among samples associated with different groups (e.g., species), a hierarchical clustering analysis on a matrix of Euclidean distances was calculated on the relative proportions of odour compounds (in % of the total blend). The hierarchical clustering analysis (Unweighted Centroid Clustering or UPGMC) was performed with the PVCLUST package (version 1.3–2;Suzuki and Shimodaira 2006) using the McQuitty method in R. Finally, the contribution of primary scent compounds within species was investigated using principal components analysis (PCA) in PAST (version 2.08;Hammer et al. 2001). A variance–covariance matrix of the floral scents (relative amount) was used, and the Jolliffe cut-off value obtained provided an informal indication of how many principal components should be considered significant(Jolliffe 1986). Components with eigenvalues smaller than the Jolliffe cut-off can be considered insignificant. The coefficient of each principal component implies the contributions of each compound among populations within a species, and the positive value and negative value of coefficients suggest that they contributed to different populations. The absolute values of coefficients show their contributions.

Flowering phenology

In SM, flowering activity was recorded monthly from September 2011 to August 2014 following Newstrom *et al.* 1994. The phenological stage (flowering or vegetative) of each species studied was recorded while patrolling at least 12 km of trails throughout the forest. Only plants presenting evidence of current or previous flowering (e.g., flowers, fruits, dry fruits, or dry flower axes) were included in this study.

In SJ, features of the flowering phenology and flower duration of *Z. crinitum* were recorded by visiting the study areas monthly, from July 2012 to July 2014. The visits were intensified (weekly) during the flower period. At INP, the observations on flowers of *Z. crinitum* were carried out from 22 to 24 August 2012 and from 10 to 13 and 21 to 23 August 2013.

To test whether flowering phenology among sympatric bee-pollinated orchid species at SM is segregated or staggered in time, as the result of namely competition or partition of bee visitors, we used null model approach (Castro-Arellano et al. 2010). We used annual activities (% of flowering individuals) of the species sharing bee pollinators to run null models with a random overlap among flowering phenological patterns using the ROSARIO algorithm in the Time Overlap software (Castro-Arellano et al. 2010). The Czechanowski (IC) and Pianka (IP) indexes of temporal niche overlap were calculated for our data and for each of the 10,000 iterations of the null model. The probabilities were then calculated by testing the null hypothesis that the observed temporal niche overlap index was greater or lower than that would be expected by chance in the case of overlap or segregation, respectively.

Results

Breeding system

Breeding system of the studied species varied widely, from autonomous selfing and self-compatibility to completely self-incompatible and pollinator-dependent. *Dichaea cogniauxiana* set fruit from spontaneous self-pollination (13.39 %) but not from emasculation. However, fruit set of natural pollination (57.47 %) was comparable to fruit set of selfing by hand (47.62 %) and higher than handmade cross-pollination (37.29 %). Fruit set after hand pollination treatments and under natural pollination was significantly higher than fruit set after spontaneous self-pollination, characterizing a self-compatible breeding system in which the exposure to pollinators significantly increased fruit set. On the other hand, *D. pendula* had a completely self-incompatible breeding system, in which no fruit was produced after spontaneous self-pollination (bagged flowers) and handmade self-pollination, while fruit set after hand cross-pollination was 45.45% and under natural pollination was 5.60% (Table 1).

Pabstia jugosa and *Pro. xanthina* were self-compatible, setting 40% and 60% fruits after hand-pollination, respectively, while *Pab. jugosa* yielded 50% fruit set after hand cross-pollination and *Pro. xanthina* did not set fruits after this treatment; however, results for this species may be inconclusive due the low number of pollination-experiments performed in each treatment. *Warrea warreana* was self-compatible and pollinator-dependent, with 83.9% fruit set after hand self-pollination, 92% after hand cross-pollination and 15.38% under natural pollination, while spontaneous self-pollination did not set fruits (Table 1).

Zygopetalum crinitum had a facultative form of self-incompatibility, in which the fruit set in self-pollinated flowers dependend on the pollination treatment received by other flowers in the same inflorescence. In the SJ population, where all the controlled pollination treatments were performed in each treated individual plant, the fruit set of hand cross-pollinated flowers was 46.87 % while spontaneous selfing and hand self-pollinated flowers yielded no fruits. However, in the SM population, for the individual plants that received only hand self-pollination (13 flowers in 4 individual plants) 38.46 % of the flowers yielded fruits, while none of the self-pollinated flowers from individuals that received both hand pollination treatments set fruit, with an overall fruit set after hand self-pollination of 9.21 %. Under natural conditions, fruit set of *Z. crinitum* was 3.04 % at SJ and 6.80 % at SM (Table 1). Thus, a biotic vector for pollen transfer is needed in both populations.

Zygopetalum maculatum presented a self-compatible breeding system, with fruit set after hand self-pollination similar to that of hand cross-pollination, 79.31 % and 78.33 % respectively, but only 4.88 % under natural pollination, and lack of fruits after spontaneous self-pollinations (Table 1).

Zygopetalum maxillare also presented a self-compatible breeding system, with similar fruit set under natural pollination in both study sites (INP and SM). However, spontaneous self-pollinations were observed to occur in SM, but not in INP. Noteworthy, ovary growth similar to that of initial fruit set occurred in emasculated flowers in which the inflorescence had been treated with hand pollination treatments in SM (Table 1).

Floral biology, resources and pollinators

Dichaea spp. are characterized by the lack of pseudobulbs and the presence of single-flowered inflorescences bearing small (less than 3 cm) flowers with anchor-shaped lips. *Dichaea cogniauxiana* presents cream flowers punctuated in wine (Fig. 1A and B). *Dichaea pendula* presents flowers with tepals that are primarily coloured cream-white with deep violet spots, and the lip is inversely coloured in deep violet interspaced with cream-white spots (Table 2, Fig. 1C and D). *Pabstia jugosa*, *Pro. xanthina* and the studied *Zygopetalum* present ovoid to elliptic pseudobulbs and inflorescences varying from single-flowered in *Pro.*

xanthina or 1-4 flowered in *Pab. jugosa* to 5-8 flowered in *Zygopetalum* spp. *Pabstia jugosa* presents white-cream flowers with the lip and inner tepals showing dark-violet stripes (Fig. 1E). *Promenaea xanthina* presents yellow flowers with wine-brown spots in the lip (Fig. 1F). *Warrea warreana* has white to cream flowers with a yellow and deep-wine coloured lip (Fig. 1G), it grows exclusively on the soil of old-growth or pristine ombrophilous dense forests, forming populations of hundreds of individuals. This species presents plicate and relatively broad leaves resembling those of some palm seedlings and its fusiform pseudobulbs grow close together while the roots develop in between the litter and the soil layers of the forest ground. All the *Zygopetalum* spp. are characterized by outer and inner tepals coloured in green and brown with the lip varying from white with thin violet stripes in *Z. crinitum* to broader violet-lilac stripes in *Z. maculatum* and an continuous gradient of lilac to violet in *Z. maxillare* (Fig. 1H-M). Flower morphology of *Z. crinitum* differs from the other species studied by the presence of conspicuous trichomes on the adaxial surface of the lip (Fig. 1H). The flowering individuals of *Z. crinitum* produced from one to four inflorescences with five to eight flowers. *Zygopetalum maculatum* individuals presented from 1-2 inflorescences with 2-8 flowers each, while *Z. maxillare* produced one to 18 inflorescences with 1-8 flowers.

Dichaea pendula and *D. cogniauxiana* offered chemicals and developing ovules as floral resources to their pollinators, which were used by male euglossine bees and weevils, respectively. According to the Fehling's reagent test and field verifications using microlitre syringes, *Pab. jugosa, Pro. xanthina, W. warreana*, *Z. crinitum* and *Z. maculatum* and *Z. maxillare* did not offer nectar as floral resource. Hitherto, no other floral resources directed to pollinators could be identified in *Pabstia*, *Promenaea, Warrea* and *Zygopetalum* species. *Warrea warreana*, *Z. crinitum* and *Z. maculatum* and *Z. maxillare* were pollinated by deceit by large bees (workers of *Bombus* spp., female *Xylocopa* sp. and *Centris confusa*). Conversely the resource-offering *Dichaea* flowers lasted 2-4 d while the other studied species without floral resources lasted 6-60 d. Individual flowers of *Pab. jugosa*, *Pro. xanthina*, *W. warreana, Z. crinitum* and *Z. maxillare* lasted at least 6 d under natural conditions and up to 14 d in greenhouse. Flowers of *Z. maculatum* had longest flower lifespan, with flowers lasting at least 10 d under natural conditions and 60 d when bagged in natural habitat or cultivated in the greenhouse (Table 2).

Dichaea cogniauxiana did not present any usual floral resource (such as nectar, oils, perfumes) and the only animals to pollinate flowers in the populations studied were females of *Montella* sp. nova weevils (Coleoptera, Curculionidae, Baridinae). Additionally, the observed spontaneous self-pollination in *D. cogniauxiana* can be attributed to rain-assisted self-pollination, as it is suggested by the occasional accumulation of water covering the stigmatic region and part of the anthers (Fig. 1B). *Dichaea pendula* attracts both *Montella* sp. n. and orchid-bees (Hymenoptera, Apidae, Euglossini), although only the bees are capable of pollen transfer between individual plants. Because *D. pendula* is self-incompatible and the weevils only promote self-pollination, solely orchid-bees contribute to fruit set in this species. Interestingly, in both *D. cogniauxiana* and *D. pendula*, female weevils of *Montella* sp. n. self-pollinate flowers by manipulating pollinaria and transferring it from anthers to the stigma in a newly described behaviour of active pollination. Afterwards, the weevils oviposited inside the stigmatic cavity, repeating this behaviour in unpollinated flowers only (C.E.P. Nunes, in prep.).

In *Pab. jugosa* and *Pro. xanthina,* no pollinator or pollination event was observed. We recorded *Montella* sp. as floral visitors for all the species studied. Specifically in *Pro. xanthina* (Fig 1F), female weevils laid eggs directly into the ovaries of unpollinated flowers and their larvae fed on the ovary tissues of the flower, leading to flower abortion and fall-down on the forest soil. After feeding on almost all the ovary internal tissues, these larvae abandoned the decomposing flower and pupate in the litter. Thus, due to the different reproductive behaviour, we infer that the *Montella* species interacting with *Pab. jugosa* and *Promenaea* are different from the species actively pollinating *Dichaea* species.

Warrea warreana was solely pollinated by *Bombus brasiliensis* (Apidae, Bombini), which entered into the tube formed by the lip and column and removed pollinaria while crawling backwards out from the tube. In this case, pollinaria got attached to the scutellum of the bee (Fig. 2X and video in electronic supplementary material). Flowers of *Z. crinitum* were exclusively pollinated by *Xylocopa* sp. females at SJ and SM study sites (Fig. 1I and video in electronic supplementary material). Pollinators generally visited one flower per inflorescence from 12:00 to 16:00 h. Each visit lasted 5–18 s. At SJ, during the observations on flowers of *Z. crinitum*, 13 visits by *Xylocopa* sp. were recorded, whereas at SM only one visit of *Xylocopa* sp. to three flowers from an individual plant was recorded. Furthermore, five pollinarium removals' were observed at SJ. Visits started with the bee landing on the lip, crawling towards the cavity formed by the lip and the column, inserting the head into this cavity, reaching its end and finally removing the pollinarium while getting out of this cavity (see video in electronic supplementary material). Pollinarium removal occurred when bees contacted the viscidium with their head (Figs. 1I and 2X), where the pollinaria got attached (the back of the head) and they were always removed with the anther cap. Lastly, *Z. crinitum* was visited but not pollinated by male orchid-bees (see video in electronic supplementary material). *Zygopetalum maculatum* was pollinated by workers of *Bombus* sp. and *B. brasiliensis*, which removed pollinaria by inserting the head into the cavity formed by the lip and the column reaching its end and removing pollinaria while getting out or this cavity in a similar way to *Xylocopa* bees in *Z. crinitum*. In the case of *Z. maculatum*, pollinaria got attached to the top of the head of the pollinator (Fig 2X). *Zygopetalum maxillare* was visited and pollinated by the solitary bees *Centris confusa* (Apidae, Centridini) at SM and INP; however, in INP, the bumble-bees *B. brasiliensis* and *B. morio* also made legitimate visits to their flowers, but no pollinaria removal or carrying by these bees was observed (Fig. 1L and M). Noteworthy, *Montella* sp. larvae also fed on ovary tissues of *Z. maculatum, Z. crinitum* and *Pab. jugosa* and adult weevils fed on flowers of all the species studied.

Floral scents

In field conditions, all species studied released a sweet floral fragrance during hottest hours of the morning and early afternoon (10:00h-13:30h), except for *D. pendula* which presented a strong smell of clove by the same period and for *Z. crinitum* which emitted a strong sweet floral scent for all day long. Nevertheless, during most of the observation time and eventually the period from 10:00h to 13:30h, flowers of *D. cogniauxiana* and *Pro. xanthina* did not release perceptible scent.

We identified 54 VOCs from the headspace samples of the eight *Zygopetalinae* orchid species: fatty acid derivatives (7), benzenoids (13), monoterpenoids (8) and sequiterpenoids (26) (Table S2). Each species emitted a distinct VOC blend, and multiple response permutation procedure (MRPP) indicated that floral scents (relative amounts, in %) were significantly differentiated among species (MRPP, A = 71.04, $d_{obs} = 24.53$, $d_{exp} = 84.69$,$p < 0.001$).Similarly, the ANOSIM showed significant difference among Zygopetalinae species (ANOSIM, R = 0.9616, P < 0.01).

The hierarchical clustering analysis (UPGMC) resulted in four main clusters (more than 95% of support in AU resampling): one major cluster composed by the samples of *Pro. xanthina, Z. crinitum*, *Z. maculatum* and *Z. maxillare* (majorly pollinated by large bees: bumble-bees and *Xylocopa* sp.), a cluster composed by *D. cogniauxiana* and *W. warreana* and two monospecific clusters composed namely by *D. pendula* and *Pab. jugosa.* The samples of the euglossine-pollinated *D. pendula* did not cluster with any other species, while *Pab. jugosa* formed a weakly supported (83% support) cluster with the major cluster formed by

Promenaea and *Zygopetalum* species (Fig. 3).The analysis of intraspecific levels of variance dispersion with Euclidean distances among floral scent samples showed that there were significant differences within species (ANOVA $F_{1,959}$= 5.35, $p = 0.02$).

According to the ICA, the sesquiterpene β-farnesene is significantly associated with *D. cogniauxiana* ($p = 0.005$), while the exclusive 2-methoxy-4-vinylphenol dominates the fragrance of *D. pendula* and *cis*-α-bisabolene is significantly associated with *Pab. jugosa* ($p = 0.01$). The following 14 compounds are significantly associated to *Z. crinitum*: 1,2,3,4-tetramethoxybenzene ($p = 0.005$); 1,2,3-trimethoxybenzene ($p = 0.005$); 1,2-dimethoxybenzene ($p = 0.005$), benzoic acid, 2-hydroxy,methylester ($p = 0.005$); benzoic acid, 2-methoxy, methylester ($p = 0.005$); benzylacetate (p= 0.005); dodecanal ($p = 0.005$); hexadecane ($p = 0.005$), methylanthranilate (p= 0.005); tetradecanal ($p = 0.005$); farnesyl cyanide (p= 0.005); linalool (p= 0.005); 1-pentanol, 2-ethyl, 4-methyl (p= 0.015) and *trans*-nerolidol (p= 0.030). The following three compounds are significantly associated to *Z. maxillare*: allo-aromadendrene (p= 0.005), longicyclene (p= 0.005) and longifolene (p= 0.025). No specific compound is exclusively associated to *Pro. xanthina, Z. maculatum* and *W. warreana.*

Furthermore, there are combinations, *i.e.*, associations of a given compound to a group of species. In this sense, β-cubebene is associated to both *D. cogniauxiana* and *W. warreana* ($p = 0.005$), α-longipinene to both *Pab. jugosa* and *Z. maxillare* ($p = 0.001$), α-bergamotene to both *Pro. xanthina* and *Z. maxillare* ($p = 0.045$) and α-farnesene to both *Z. crinitum* and *Z. maculatum* ($p = 0.001$). *Trans*-α-bergamotene is associated to *D. cogniauxiana*, *Pro. xanthina* and *W. warreana* ($p = 0.008$), *cis*-β-ocimene to *Pro. xanthina*, *Z. crinitum* and *Z. maculatum* ($p = 0.001$) and *cis,trans*-α-farnesene to *W. warreana, Z. crinitum* and *Z. maculatum* ($p = 0.001$). Additionally, *trans*-β-ocimene is associated to the studied *Zygopetalum* species (p =

0.001) while β-myrcene is associated to the studied *Promenaea* and *Zygopetalum* species (p = 0.001).

Chemical composition of VOCs by PCA showed clear separation among Zygopetalinae species. PC1 explained 32.02% of the variance, while PC2 only 19.28% (Figs. S1 and S2). The sesquiterpenes β-Farnesene, β-Cubebene and *trans*-α-Bergamotene contributed exclusively to *D. cogniauxiana* with absolute loadings of 0.62, 0.17 and 0.09, respectively. Both 2-methoxy-4-vinylphenol and 4-hydroxy-3-methylacetophenone contributed solely to *D. pendula*, with low absolute loadings of namely 0.04 and 0.01. The VOC β-myrcene contributed to *Pro. xanthina* and the studied *Zygopetalum*, and the absolute loading accounted for 0.74. Further, *trans*-β-ocimene contributed to *Pab. jugosa*, *Pro. xanthina*, *Z. maculatum* and *Z. maxillare* and had an absolute loading of 0.13, while *cis,trans*-α-Farnesene contributed in small proportions to *D. cogniauxiana* and the *Zygopetalum* species, but in a high proportion to *W. warreana*, with an absolute loading of 0.05 (Fig. S2, Table S2).

Flowering phenology

The species studied present an annual flowering pattern, with the flowering occurring from two (e.g. *Dichaea* species) to six months (e.g. *Z. maculatum* in 2013) in the year (Fig. 1). *Dichaea cogniauxiana* flowered from December to February, meanwhile *D. pendula* flowered from November to January. *Pabstia jugosa* flowered between September and October and, *Pro. xanthina,* from September to December. *Zygopetalum crinitum* presented flowers from June to September. *Warrea warreana* flowered from December to April. *Zygopetalum maculatum* has the most extended flowering period (ranging from February to August in 2012) and *Z. maxillare* present flowers in the period from March to August, overlapping several months with the former species. These three bumble-bee-pollinated species have significantly overlapping flowering periods (IP = 0.82; IC = 0; $p_{(\mathrm{IP} \geq \mathrm{observed})}$ = 0.019; $p_{(\mathrm{IC} \geq \mathrm{observed})}$ = 0.017)(Fig. 4).

Discussion

Breeding system

The great variation in the breeding system of sympatric species of Zygopetalinae reflects the diversity of reproduction strategies of the vast family Orchidaceae. Except for *D. cogniauxiana*, that produces as many fruits under natural conditions as after hand cross-pollination, all the species studied experience pollen limitation, producing more fruits with hand cross-pollinations than under natural pollination (Tremblay et al. 2005).

The noteworthy occurrence of apparent initial fruit set in emasculated flowers of *Z. maxillare* may be attributed to the hormonal stimulation (likely auxins) provided by pollinated flowers in the same inflorescence of the emasculated flowers. However, further studies examining the quantity and the quality of the seeds in those treatments are necessary to determine whether the observed phenomenon constitutes agamospermy.

Pollination limitation is common in orchids occurring along Atlantic forest (Pansarin et al. 2006, 2008; Pansarin and do Amaral 2009; Wolowski et al. 2014; Amorim et al. 2014; Nunes et al. 2015).Thereby, the fact that *D. cogniauxiana* does not present any evidence of pollination limitation may be attributed to the selfing and autonomous autogamy breeding system, as well as to the abundance, constancy and efficiency of the *Montella* sp. n. weevils as pollinators, which may guarantee that fruit set under natural pollination is higher than fruit set resulting from hand self-pollination, for instance. Furthermore, once epiphytic orchids may have its fruit set restricted by resource limitation over time (Meléndez-Ackerman et al. 2000), more specific tests are needed to verify whether the trend observed for *D. cogniauxiana* is consistent for more than one reproductive season.

Floral biology, resources and pollinators

Regarding flower lifespan, we can make a clear distinction between the resource-offering and non-offering (deceptive) species studied. Flowers of the resource-offering *Dichaea* lasted no longer than four days, while flowers of the deceptive *Pabstia*, *Promenaea*, *Warrea* and *Zygopetalum* species lasted at least one week. Noteworthy, flowers of *Z. maculatum* bagged *in situ* had an extremely long flower span (up to 60 d). Thereby, the studied resource-offering flowers have a lifespan compatible with other flowering plants, while the deceptive ones have their flower lifespan longer or much longer than the average for angiosperms (Primack 1985). Hence, longer flower lifespan is considered to be a feature of deceit-pollinated orchid species (Ashman and Schoen 1994; Pansarin et al. 2008; Vale, Rojas, et al. 2011). However, floral longevity of individual plants may be influenced by biotic and abiotic factors, such as pollination events and the plant resource status (Parra-Tabla et al. 2009).

According to the clustering analysis of floral scent, the main group of species is composed by *Pab. jugosa*, *Pro. xanthina, Z. crinitum*, *Z. maculatum* and *Z. maxillare*, the latter three pollinated by large bees, namely bumble-bees and *Xylocopa* sp. This fact, together with the information that *Pab. jugosa* may naturally hybridize with the *Xylocopa*-specialised *Z. crinitum* (Hoehne 1933), allows us to hypothesize that this species may eventually be pollinated by *Xylocopa*. However, the absence of benzenoids (characteristic of the scent of other *Xylocopa*-specialised flowers) in the floral scent and the large flowers of *Pab. jugosa* suggest that this species has another system of pollination by deceit or sensorial exploitation involving large bees.

Additionally, *Pro. xanthina* is merged together with *Z. maculatum* and *Z. maxillare* in a single well supported cluster. As the two *Zygopetalum* species are both pollinated by large

Apidae bees, *Bombus* spp. workers and *Centris confusa*, it is possible to predict that *Pro. xanthina* might also be pollinated by a large Apidae, perhaps naive workers, again characterizing a deceit-pollination system.

Since *Bombus* spp. workers visit three out of the eight studied species, namely *W. warreana*, *Z. maculatum* and *Z. maxillare,* and are the most likely pollinators of *Pro. xanthina*, pollinator-sharing could lead to hybridization among those species, once they all may interbreed artificially, as demonstrated by several horticultural hybrids among these genera. The list of artificial interspecific breeding includes hybrids involving all the *Zygopetalum* species studied and intergeneric hybrids between *Zygopetalum* and *Promenaea* or involving *Zygopetalum* and *Warrea*(The Royal Horticultural Society 2014). However, several barriers may prevent the hybridization in natural conditions. For instance, the location of pollinaria deposition on the body of bumblebees may differ between the co-flowering *Z. maculatum* and *Z. maxillare*, of which pollinaria may attach to different parts of the head of the bee, and *W. warreana,* of which pollinaria attach on the thorax (Fig. 2). In addition, *Pro. xanthina,* which could also be pollinated by bumble-bees, emit flowers in a time of the year when neither any *Zygopetalum* nor *W. warreana* present flowers, what imposes a temporal barrier against natural hybridization between *Pro. xanthina* and both *Zygopetalum* and *Warrea* studied. In the case of the co-flowering pollinator-sharing *Z. maculatum* and *Z. maxillare*, the mechanisms which would avoid interspecific breeding are unclear and may be related to slight differences in the position on which pollinaria attach to the head of the bees or to differences in pollinaria morphology between the two species. Further observations and experiments about pollinaria attachment on shared pollinators, hybridization and hybrids viability are necessary to clarify such mechanisms (Roubik and Ackerman 1987; Singer and Sazima 2004).

Male orchid-bees (*Euglossa* spp.) visited but did not pollinate the flowers of the *W. warreana*(H. Heider, personal communication; Fig. 1G, inset) and *Z. crinitum*(video in electronic supplementary material). In *W. warreana*, the behaviour of *Euglossa* could not be recorded in detail, while in *Z. crinitum*, *Euglossa* sp. exhibited a typical fragrance-collecting behaviour (Vogel 1966; van der Pijl and Dodson 1969; Faegri and van der Pijl 1979). The occurrence of visiting and fragrance collecting by male orchid-bees in Zygopetalinae flowers pollinated by deceit by other Apidae provide the first record of a close phylogenetic relation (within the level of subtribe) between pollination by deceit and pollination by male orchid-bees in which fragrance is the floral resource. Additionally, these two facts allow us to ask whether (1) pollination by male orchid-bees would have evolved from deceit-pollinated taxa or (2) pollination by deceit would have evolved by previously orchid-bee-pollinated taxa. In the situation of hypothesis (1), relatively simple changes in the flower parts emitting fragrances collected by the male orchid-bees could lead to the function of these floral visitors as pollinators. In hypothesis (2), changes in the composition of the fragrance and floral morphology could enhance namely the attraction of food-foraging Apidae bees and its function as pollinators of Zygopetalinae orchids.

Adult *Montella* sp. weevils visited the flowers of all the populations from all the species studied at SM study site. In addition, unpollinated damaged flowers, lacking the ovary tissues, were found amongst flowers of the big-flowered *Pab. jugosa*, *W. warreana*, *Z. crinitum* and *Z. maculatum*. Furthermore, interactions between *Montella* weevils and flowers were observed at SM in the fragrant bee-pollinated orchid species *Grobya amherstiae*, *Bifrenaria harrisoniae* and *Gomesa crispa* (Mickeliunas et al. 2006; C.E.P. Nunes personal observations). Together with previous observations from Mickeliunas et al. (2006) on *G. amherstiae* interaction with *Montella* weevils at SJ, the findings of these works suggest that *Montella* spp. interactions with flowers may result in different outcomes to plants, varying from parasitic destructive florivory to mutualistic active pollination with consumption of only part of the pollinated flowers (Mickeliunas et al. 2006; C.E.P. Nunes, unpublished data). Abiotic and temporal factors may influence the distribution of the interacting plant-pollinators, as predicted by the geographic mosaic theory of coevolution (Thompson 2005; Thompson and Fernandez 2006). Further, the outcome of the interaction between orchid flowers and these florivory/pollinating weevils to the plants would depend on several plant traits, such as breeding system, flower morphology and flower size, as observed in other pollination systems involving ovule consumption (Ibanez 2009; Ibanez et al. 2009; Wang et al. 2010).

Floral scents

The studied species emitted particular floral bouquets according to UPGMC, which separated in distinct groups, suggesting that floral fragrance determine the pollination system. Exceptionally, *D. pendula* had its floral scent dominated by 2-methoxy-4-vinylphenol, an unusual floral VOC which was responsible for its separation from all the other species studied. On the other hand, *D. cogniauxiana* samples formed a separate group with several typical floral VOCs, with few relations to the bouquets of the other Zygopetalinae species studied. Thus, strictly in the context of this work, the floral scent composition of this species may be associated to the attraction of and pollination by *Montella* sp. weevils. Due to the presence of floral VOCs common to other bee-pollinated taxa in the floral scent of *D. cogniauxiana* (e.g. Bergamotene, β-Cubebene, Zingiberene and Farnesene isomers, Table S2) (Knudsen and Mori 1996; Knudsen et al. 2006; Gong et al. 2014), we hypothesize that this species is also pollinated by bees, at least in other populations than the studied in this work. Thereby, more observations on floral visitors of *D. cogniauxiana* in the present study area and in other areas of its distribution are needed to confirm the existence of such a specific association.

Also remarkable was the composition of the fragrance of the *Xylocopa*-specialized *Z. crinitum,* which contained several distinctive VOCs, including terpenoids, benzenoids and fatty acid derivatives composing a relatively complex bouquet previously assessed in other studies (Gerlach and Schill 1991; Kaiser 1993). VOCs identical or structurally very similar to the distinctive components of *Z. crinitum* flower scent (*e.g.* 1,2-dimethoxybenzene and 1,2,3-trimethoxybenzene) are also found in flowers of other *Xylocopa*-specialized plants such as *Passiflora edulis* (M.C. Dornelas personal communication), *Calanthe* spp. (Awano et al. 1997; Sugiura 2013) and *Vanda* spp. (van der Pijl and Dodson 1969; Kaiser 1993). In addition, some of the exclusive components of *Z. crinitum* floral bouquet (linalool, Nerolidol, Farnesyl cyanide, Farnesane, Tetradecanal in this work; Geraniol, Geranial, Nerol and others in Gerlach and Schill 1991 and Kaiser 1993) are structurally identical or very similar to known *Xylocopa* pheromones (Vinson et al. 1978; Williams et al. 1987; El-Sayed 2012). Thus, the complex and distinctive composition of the fragrance of *Z. crinitum* can be associated with pollination by exclusive sensorial exploitation of carpenter bees, conferring for this species a “hidden” pollination syndrome or chemical private niche (Chen et al. 2009; Soler et al. 2010; Lunau et al. 2011; Schäffler et al. 2015) characterized by a distinctive set of floral VOCs within flowers pollinated by large Apidae.

Furthermore, the floral VOC blends of all species studied may be composed by a set of pollinator-attracting VOCs but also by herbivore-avoiding VOCs, as the result of selective pressures of both pollinators and generalist herbivores (Andrews et al. 2007; Theis and Adler 2012; Kessler et al. 2013). As demonstrated by Kessler et al. (2013) for *Petunia* flowers, the isoeugenol, found here in trace amounts in the floral scent of *D. pendula*, could act as an herbivore deterrent. This way, the orchid-specialized herbivore weevils *Montella* sp. would have evolved strategies not only to track orchid flowers scents but also to deal with orchid chemical defences.

Our results suggest that phylogenetic relationship may explain floral fragrance composition in some clades, but not in all the species studied. For instance, the VOC *trans*-β-Ocimene was common in the floral scent of *Pab. jugosa, Pro. xanthina* and the three *Zygopetalum* species. In the same way, *cis*-β-Ocimene and β-Myrcene were common to the floral scent of *Pro. xanthina* and the three *Zygopetalum* species. Once *Pabstia*, *Promenaea* and *Zygopetalum* share closer phylogenetic affinities to each other than to the other genera studied, these compounds may be related to the closer phylogenetic affinities of these three clades (Whitten et al. 2005).However, the studied *Dichaea* species did not share any floral VOC, suggesting that specialization driven by pollinator-mediated selection by distinct pollinator groups (*i.e.* weevils, male orchid-bees and likely other bees), may have resulted in great divergence in floral fragrance composition within *Dichaea*. Though, a more comprehensive phylogeny for Zygopetalinae that would allow researchers to perform a phylogenetic analysis of floral scents in the clade is still lacking.

Although the three *Zygopetalum* species studied were pollinated by deceit by large Apidae and clustered in the same group according to their floral scents, *W. warreana* was also pollinated by deceit by large Apidae but clustered in a separate group more similar to *D. cogniauxiana* (Fig. 3). Thereby, floral scents alone cannot provide an accurate prediction of the pollinators in the species studied. However, if we take into account the flower diameter and lifespan it is possible to predict that all the studied Zygopetalinae with flowers larger than 3 cm lasting more than four days (Table 2) shall be pollinated by deceit by large bees, while the species with smaller flowers will present other pollination systems (*e.g.* pollination by male Euglossini bees).

Flowering phenology: a barrier against interspecific breeding?

Together, the species pollinated by deceit by large bees have flowering periods spanning all the year at SM. However, the *Dichaea* species, which offer resources to their pollinators, flower only during the warmest and rainiest season, when euglossine-bee pollinators, and probably also weevil pollinators are more abundant in the study area, as suggested by a previous study with Euglossini bees in a close location (Singer and Sazima 2004). Specifically, the euglossine-pollinated *D. pendula* may have its flowering phenology overlapped with the peak of abundance of its most effective pollinators, including *Euf. violacea*, an orchid-bee with known seasonal occurrence restricted to that period (Singer and Sazima 2004; Nunes et al. 2015). The flowering phenology pattern of the studied *Dichaea* species follows the pattern of most Atlantic forest tree species occurring in the studied region. Most of these plant species are visited and pollinated by insects and flower during the beginning of the warmest/rainiest season with their flowering activities significantly correlated with temperature and day length (Morellato et al. 2000; Rocha-Filho et al. 2012).

As demonstrated for other orchid species pollinated by deceit, the reproductive success may be influenced by the flowering time in relation to the flowering peak within populations and sometimes by the presence of a co-occurring resource-offering species sharing pollinators nearby the deceptive orchid (Parra-Tabla and Vargas 2004; Vale, Navarro, et al. 2011). For instance, in the deceptive *Tolumnia guibertiana* (A.Rich.) Braem (Orchidaceae, Oncidiinae), pollination in a given population is enhanced by the presence of the resource-offering *Ouratea agrophylla* (Tiegh.) Urb. (Ochnaceae) which works as the model of the mimetic orchid (Vale, Navarro, et al. 2011). Another similar mimicry system involves the pollination of *Cyrtopodium polyphyllum* (Vell.) Pabst *ex* F. Barros by oil-collecting bees (Centridini), in which the orchid use the same signals of the oil flowers of sympatric *Stigmaphyllum arenicola* C.E. Anderson (Malpighiaceae) (Pansarin et al. 2008). Mimicking nearby resource-offering species could be the strategy for *Pab. jugosa* and *Pro. xanthina.* The former two species would emit the same signals of other sympatric co-flowering species, such as the more abundant bee-visited and nectar-offering *Neomarica* spp. (Iridaceae) and *Aphelandra* spp. (Acanthaceae) (C.E.P. Nunes, personal observation; see some records on the flower visitors of *Neomarica* at https://www.youtube.com/watch?v=HBeUN7HdldY). However, further studies are necessary to test for the existence of true floral mimicry in any of the studied orchid species.

In another deceptive tropical orchid, *Myrmecophila christinae* Carnevali & Gómez-Juárez (Orchidaceae, Laeliinae), probability of pollen transfer increases further from the flowering peak, hence disruptive selection on flowering phenology acts favouring individuals that flower longer before or after the population flowering peak (Parra-Tabla and Vargas 2004). That could be the case of *W. warreana*, *Z. crinitum*, *Z. maculatum* and *Z. maxillare*, which, in some years, presented extended flowering periods (up to six months in *Z. maculatum*), more than one flowering peak or single individuals flowering completely out of the population flowering peak (*Z. crinitum* and *W. warreana*).

Reproductive isolation between the studied species with the absence of hybrids cannot be attributed to the flowering phenological patterns of the species that share pollinators, such as *W. warreana*, *Z. maculatum* and *Z. maxillare.* In the former three species, the flowering period is partially or fully overlapped and other mechanisms are expected to exist to avoid hybridization as well as competition for bumble-bees. Due to the different distances from the putative nectar source (formed by the lip in *Z. maculatum* and *Z. maxillare* and by the lateral sepals in *W. warreana*) to the viscidium, it is likely that isolation between these three species occurs by differential positioning of the pollinaria on the body of bumble-bee pollinators. In the specific cases of the co-flowering *Z. maculatum* and *Z. maxillare*, the reproductive isolation could be reinforced by differences and mismatches in pollinaria/stigma morphologies. Different positioning of the pollinaria on the pollinators bodies have been showed to be one of the mechanisms avoiding interspecific pollen-transfer in several co-flowering sympatric orchids pollinated by Euglossini bees (Roubik and Ackerman 1987; Singer and Sazima 2004).

Conclusion

Unlike what has been suggested in the literature, the orchid subtribe Zygopetalinae comprises fragrant orchids pollinated by deceit by solitary and social large Apidae. The fact that *W. warreana* and *Z. crinitum*, respectively deceit-pollinated by *B. brasiliensis* and *Xylocopa* sp. bees, are also visited by male *Euglossa* sp. (Apidae, Euglossini) foraging for chemicals suggests a possible pathway to the evolution of pollination by orchid-bee. This way, a pollinator shift to male euglossine-pollination from bee deceptive pollination or vice-versa can occur by slight changes in flower morphology or a simple change in the flower parts emitting given chemicals. Although we could not register pollinators in *Pab. jugosa* and *Pro. xanthina*, our multivariate analyses, the large size and long lifespan of the flowers suggest large bees are the most likely pollinators of these species, again characterising deceptive pollination systems or sensorial exploitation of Apidae pollinators. Thus, this study reveals the possibility of predicting orchid pollinators based on floral scent together with other floral traits, *e.g.* flower size and lifespan. In addition, this work provides novel insights on the evolution of pollination by male orchid-bees in Orchidaceae by establishing a closer phylogenetic link (below the level of subtribe) of this pollination system to pollination by deceit by other Apidae bees. Additionally, we unravel the existence of a “hidden” pollination syndrome or chemical private niche exclusive to carpenter bees characterized by a distinctive set of floral VOCs within flowers pollinated by large Apidae. However, more information on the pollination ecology of the extant Zygopetalinae (reliable information is available for only *ca.* 20 out of the 420 species in the subtribe) is needed to perform further analyses on the evolution of pollination systems in the clade, allowing a better comprehension of the origins of orchid pollination by fragrance-collecting male euglossine.

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Tables

Table 1 Controlled pollination experiments results of eight Zygopetalinae orchid species from south-eastern Brazil. Percentage of fruit set (number of fruits / number of flowers) and number of flowers (in parenthesis) treated in controlled pollination experiments at different locations in south-eastern Brazil. Data for all species were collected at Serra do Mar, with additional data for *Z. crinitum* at Serra do Japi (SJ) and for *Z. maxillare* at Itatiaia National Park (INP).

	D. cogniauxiana	*D. pendula*	*W. warreana*	*Z. crinitum*	*Z. crinitum* SJ	*Z. maculatum*	*Z. maxillare*	*Z. maxillare* INP
emasculation	0 (38) a	-	-	0 (3)	0 (32)	0 (5)	5.88 (17)**	0 (8)
spontaneous self-pollination	13.39 (112) b	0 (32)	0 (18)	0 (12)	0 (32)	0 (9)	100 (6)	0 (8)
hand self-pollination	47.62 (42) d	0 (24)	83.87 (31)	9.52 (21)*	0 (32)	79.31 (58)	15.38 (13)	22.22 (18)
hand cross-pollination	37.29 (59) c	45.45 (33)	92 (25)	88.89 (9)	46.87 (32)	78.33 (60)	46.15 (13)	100 (9)
natural pollination	57.47 (395) d	5.6 (125)	15.38 (26)	6.8 (103)	3.04 (197)	4.88 (205)	17.71 (96)	16.66 (549)
reproductive system (in relation to self-fertilization	compatible	incompatible	compatible	incompatible*	incompatible	compatible	compatible	Compatible

Different letters in each column represent significant differences in the Chi-square ($p \leq 0.05$) test performed when applicable.

* Only inflorescences without cross-pollinated flowers set fruits after self-pollination.

** Only inflorescences with hand pollinated flowers set fruits after emasculations.

Table 2 Habit, flower size and lifespan, floral resources and animal pollinators of eight Zygopetalinae orchid species from south-eastern Brazil.

	D. cogniauxiana	*D. pendula*	*Pab. jugosa*	*Pro. xanthina*	*W. warreana*	*Z. crinitum*	*Z. maculatum*	*Z. maxillare*
Habit	epiphyte	epiphyte	epiphyte	epiphyte	terrestrial	epiphyte	terrestrial	epiphyte
Floral resource	developing ovules	perfume	absent	absent	absent	absent	absent	absent
Flower diameter (cm)	< 1	< 2	> 4	> 3	> 3	> 4	> 3	> 3
Flower lifespan (days)	2-4	2	> 10	> 6	> 6	> 14	> 14	> 14
Pollinators	*Montella* sp. n. weevils	Male euglossine bees and *Montella* sp. n.	Unknown	Unknown	*Bombus brasiliensis* bee	*Xylocopa* sp. bee	*Bombus* spp. bees	*Bombus* spp. and *Centris confusa* bees

Figures

Fig. 1 Flowers and some of the pollinators of the eight Zygopetalinae species studied in south-eastern Brazil. (A) *Dichaea cogniauxiana* being actively pollinated by a female *Montella* sp. n. weevil, which removes the pollinaria (arrow) and carries it to the stigmatic cavity. (B) Flower of *D. cogniauxiana* with the anthers and stigmatic cavity involved by a water drop (arrow), which may enhance self-pollination in this species. (C) *Dichaea pendula* flower and (D) its pollination by *Eufriesea violacea*, which carries the pollinaria on the head (arrow). (E) *Pabstia jugosa* bearing two inflorescences. (F) Flower of *Promenaea xanthina* being visited by *Montella* sp. weevil which, in this case, do not pollinate, only feeds on flower tissues. (G) Flower of the bumble-bee pollinated *Warrea warreana*, a species visited but not pollinated by an orchid-bee (inset). (H) Inflorescence of the carpenter bee-pollinated *Zygopetalum crinitum* and (I) a female carpenter bee (*Xylocopa* sp.) carrying a pollinarium on its head (arrow)(J)The bumble-bee-pollinated flower of *Z. maculatum.* (K) Inflorescence of *Z. maxillare* and its flowers being visited by (L) the bumble-bee (*Bombus* sp.) and (M) the solitary bee *Centris confusa*. Scale bars = 1 cm.

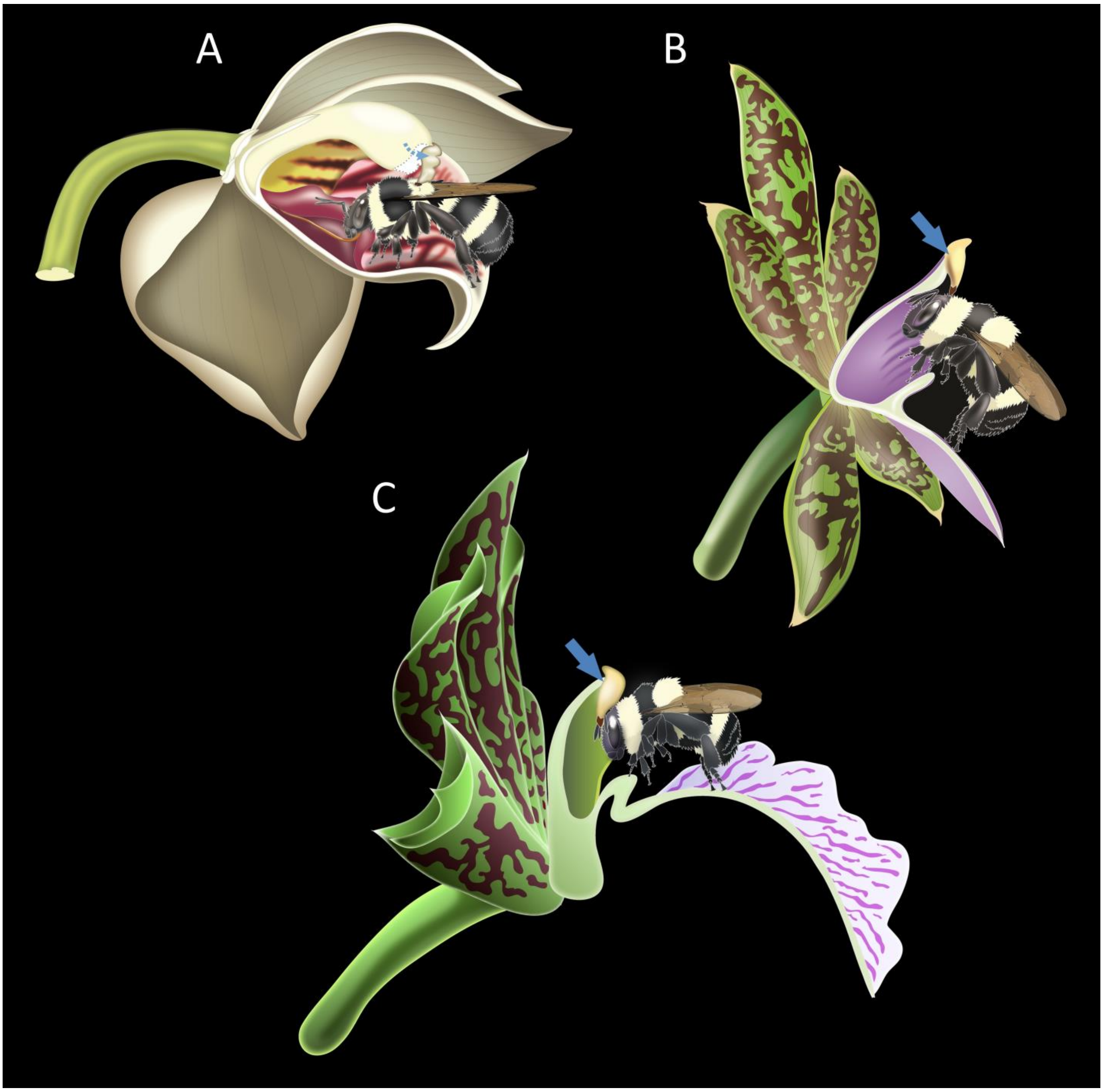

Fig. 2 Details on mechanisms and positioning of pollinarium on the body of pollinators in bumble-bee-pollinated Zygopetalinae species studied in south-eastern Brazil. In all the three species, the bee removes pollinaria when getting out of the cavity formed by the lip and the column. However, in (A) *Warrea warreana* pollinaria attaches onto the thorax, in (B) *Zygopetalum maxillare* it attaches onto the back of the head, while in (C) *Z. maculatum* it attaches on the head front.

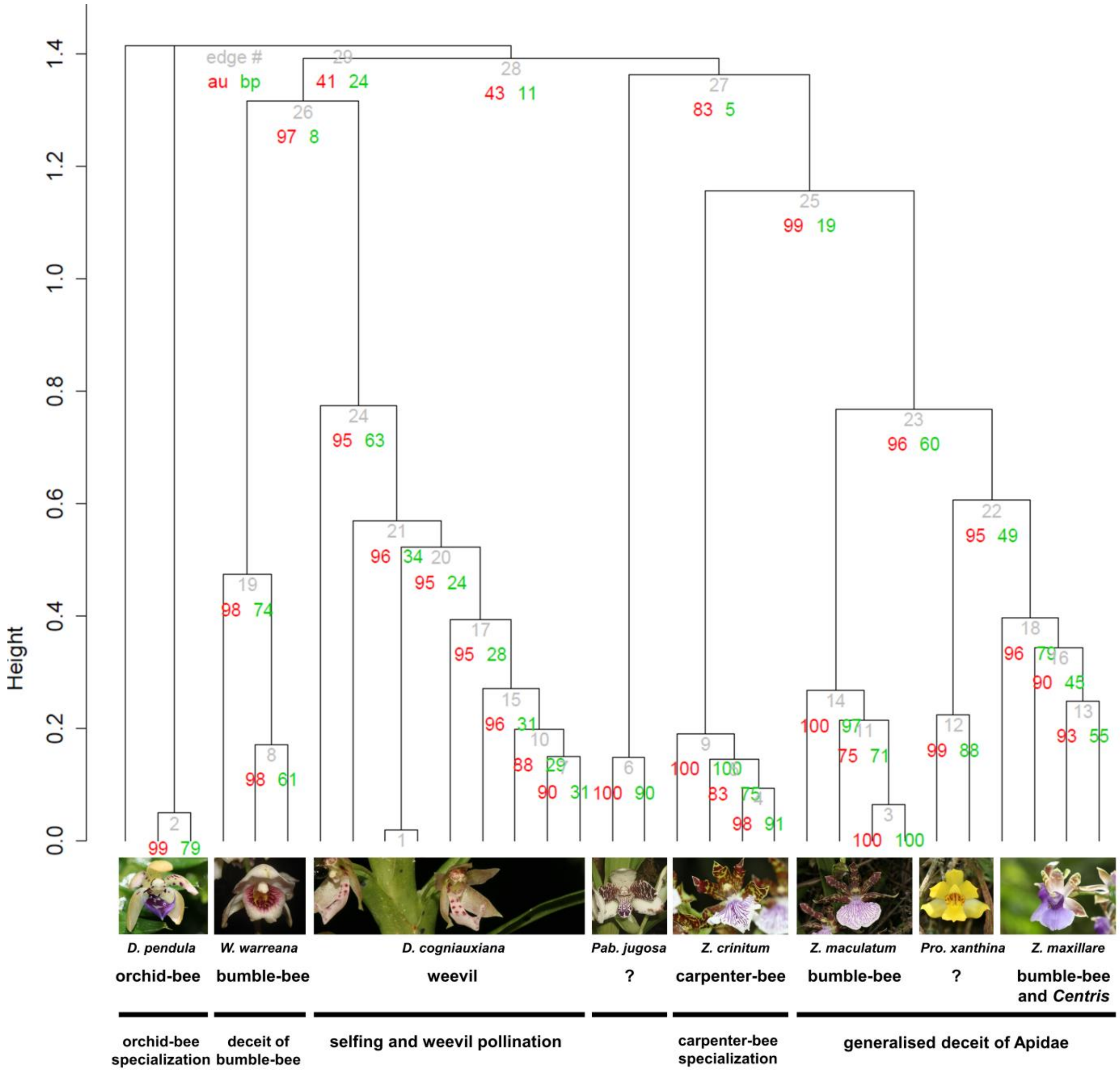

Fig. 3 Floral scent differentiation among the eight Zygopetalinae species studied in south-eastern Brazil: cladogram of the hierarchical clustering analysis (UPGMC using Euclidean distances) of floral scent differentiation based on a matrix of the relative proportions of odour compounds (in % of total blend). The values in red and green are the probabilities (in %) obtained from two resampling methods, respectively, Approximately Unbiased (AU) and Bootstrap Probability (BP). The terminal branches represent each sample and are linked to images of the species as in Fig 1. The groups well supported in AU (> 95%) and the respective pollinators and pollination systems are identified.

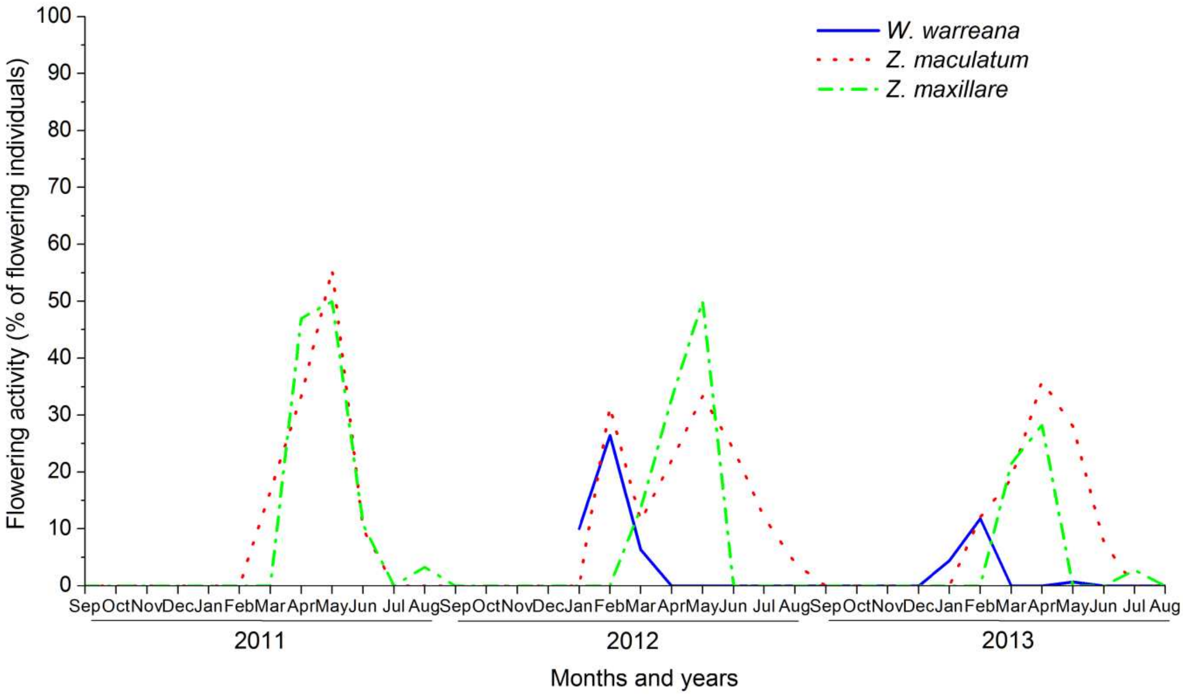

Fig. 4 Flowering phenology patterns of the Zygopetalinae orchid species sharing bee pollinators occurring sympatrically at Serra do Mar State Park, south-eastern Brazil, during the period ranging from August 2011 to September 2013. *Warrea warreana*, *Zygopetalum maculatum* and *Z. maxillare* are pollinated by the bumble-bees *Bombus brasiliensis* and *Bombus* sp..

Supplementary material

Floral scents and pollinators of Zygopetalinae orchids: diverse pollination systems in a group previously only known to be euglossine-pollinated

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Figures

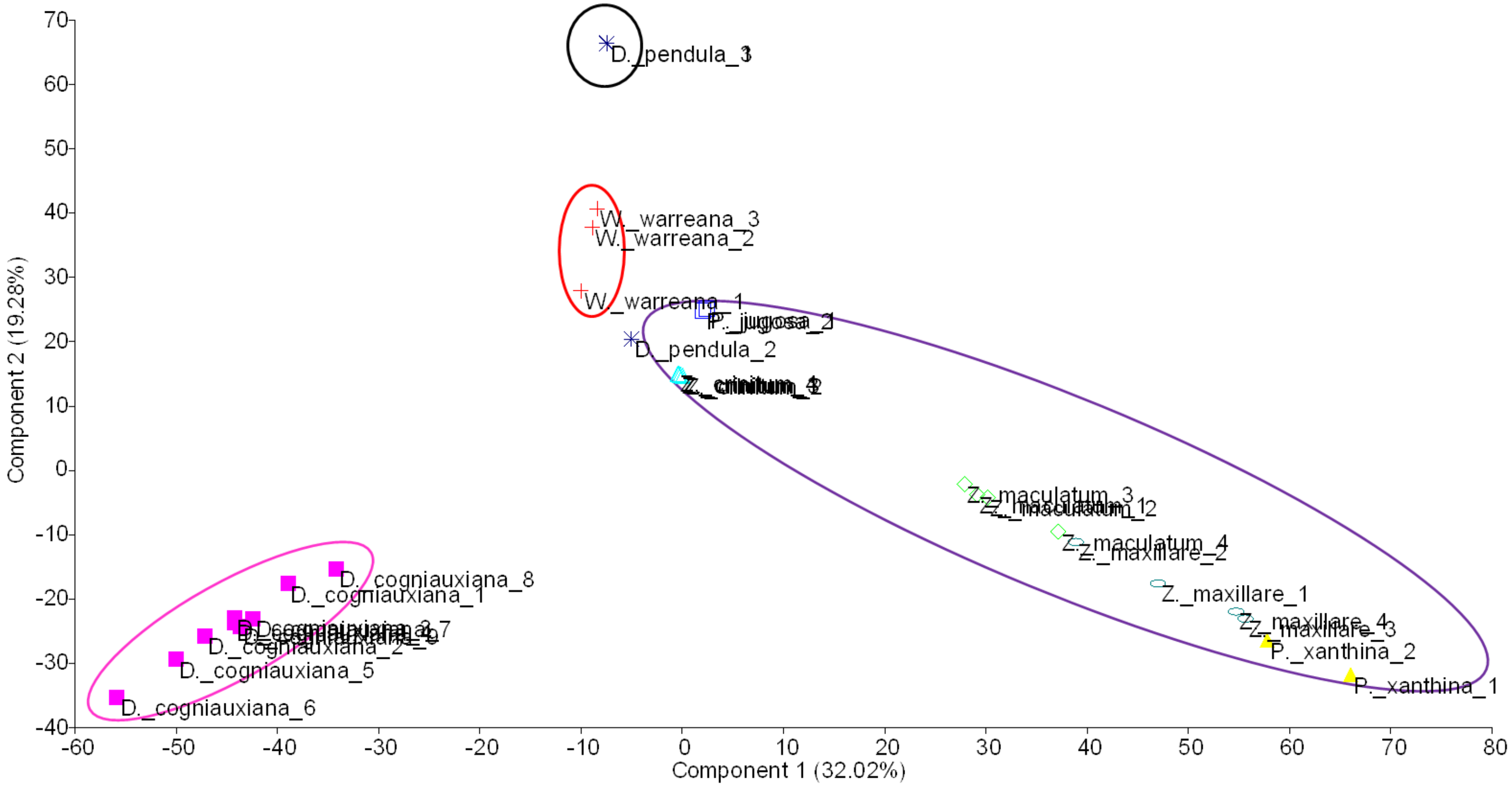


Fig. S1

Distribution of the samples in the principal components analysis (PCA) using floral scent data of eight Zygopetalinae orchid species from the Atlantic forest of south-eastern Brazil. The percentage of variance explained by each component (1 and 2) is listed in parenthesis. Each ellipse delimitates a group of samples in accordance to the hierarchical clustering analysis (UPGMC).

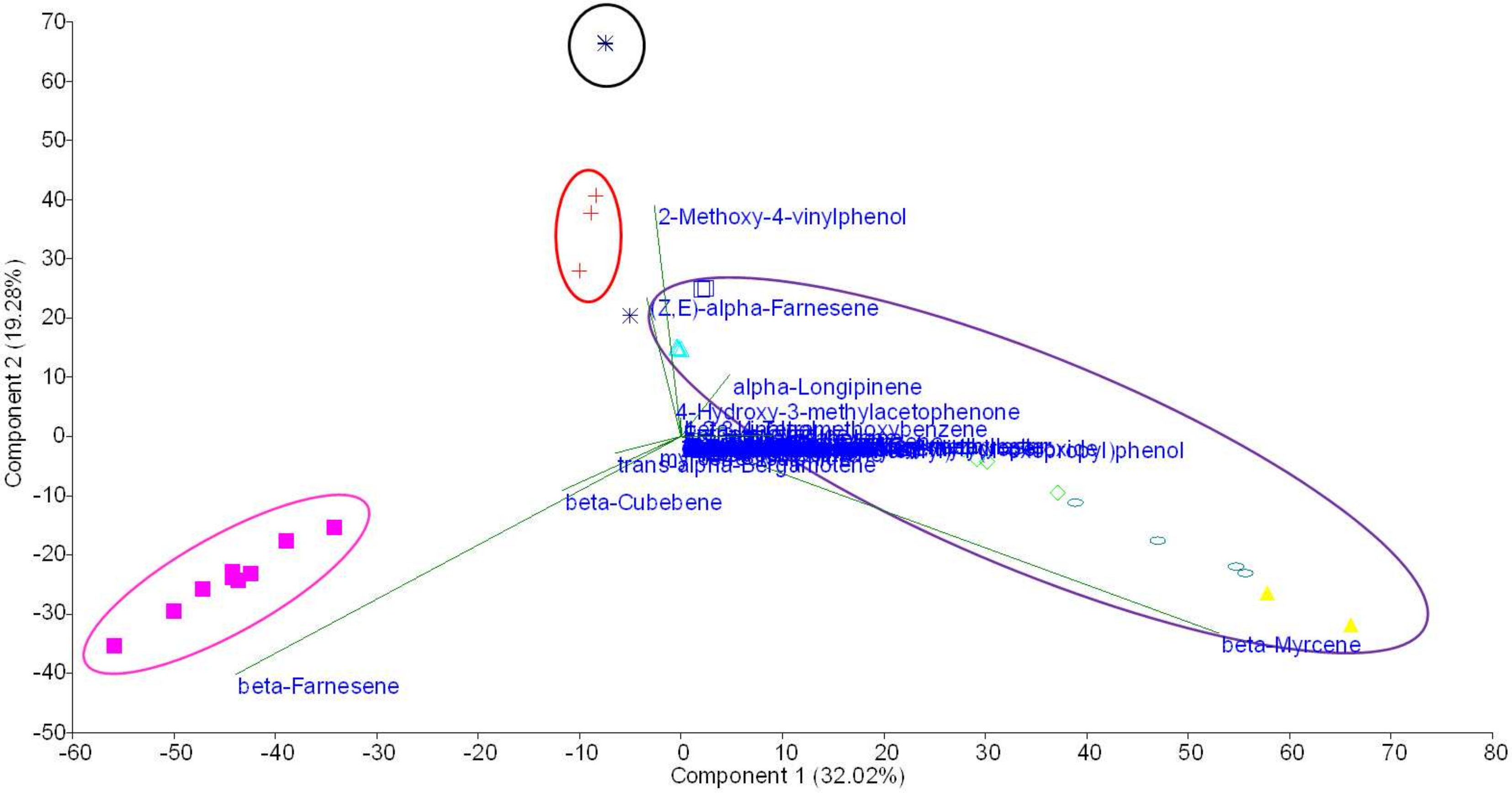


Fig. S2

Principal components analysis (PCA) using floral scent data of eight Zygopetalinae orchid species from the Atlantic forest of south-eastern Brazil. The proportional contribution of each floral volatile to the total variance is represented by the lines departing from the centre of each axis. The percentage of variance explained by each component (1 and 2) is listed in parenthesis. Each ellipse delimitates a group of samples in accordance to the hierarchical clustering analysis (UPGMC).

Tables

Table S1 Number of individuals and flowers (in parenthesis) used in the controlled pollination experiments and hours of focal observations in eight Zygopetalinae orchid species from the Atlantic forest of south-eastern Brazil. We present numbers for all the species studied at Serra do Mar, and discriminate the numbers for *Z. crinitum* and *Z. maxillare* additionally studied at Serra do Japi (SJ) and Itatiaia National Park (INP), respectively.

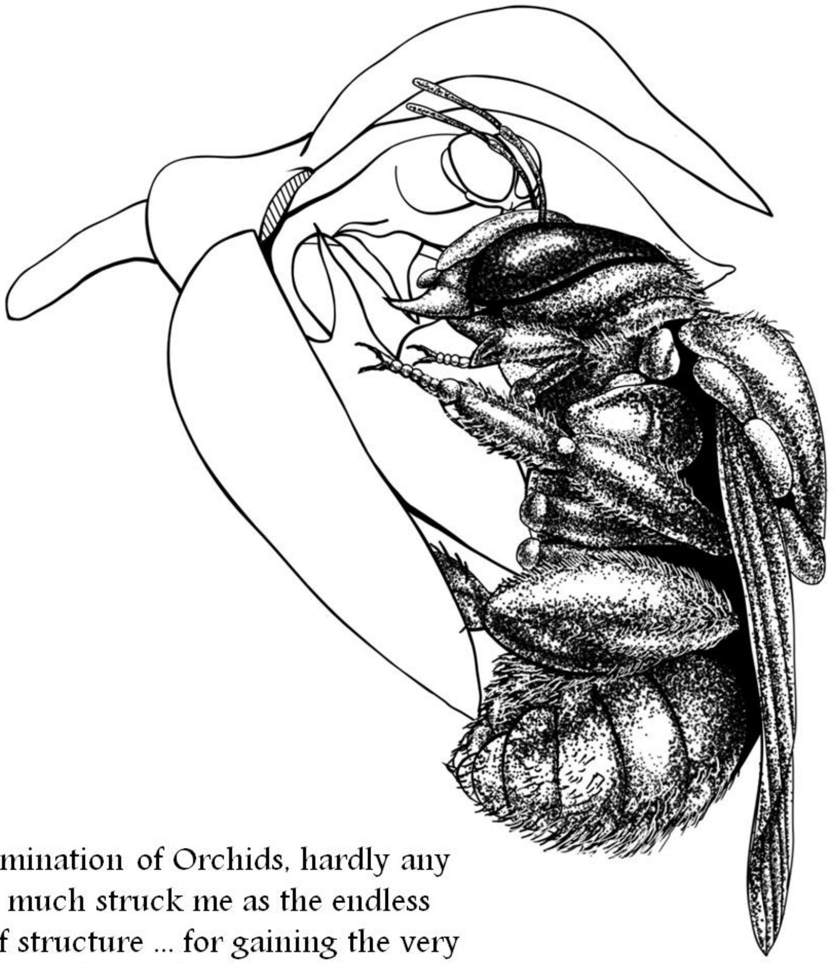
	D. cogniauxiana	*D. pendula*	*Pab. jugosa*	*Pro. xanthina*	*W. warreana*	*Z. crinitum*	*Z. crinitum* SJ	*Z. maculatum*	*Z. maxillare*	*Z. maxillare* INP
emasculation	5(38)	2(2)	-	2(4)	-	2(3)	16(32)	1(5)	4(17)**	13(8)
spontaneous self-pollination	11(112)	7(32)	-	-	3(18)	4(12)	16(32)	2(9)	1(6)	13(8)
hand self-pollination	7(42)	16(24)	2(5)	4(9)	9(31)	10(21)*	16(32)	19(58)	3(13)	13(18)
hand cross-pollination	10(59)	18(33)	4(4)	4(9)	9(25)	7(9)	16(32)	18(60)	5(13)	13(9)
natural pollination	34(395)	52(125)	3(6)	6(12)	7(26)	13(103)	15(197)	30(205)	14(96)	57(549)
Focal observations (h)	39	84	33	78	19	67	83	71	89	41

Table S2 Floral scent compounds of the eight Zygopetalinae orchid species investigated. Mean ± SD, relative amounts (%) of odour compounds in headspace fractions of *Dichaea cogniauxiana*, *D. pendula*, *Pabstia jugosa*, *Promenaea xanthina*, *Warrea warreana*, *Zygopetalum crinitum*, *Z. maculatum* e *Z. maxillare* (*tr* indicates trace amounts, i.e., compounds with concentration < 0.1 %and- indicates not detected compounds).The compounds are ordered in classes, and listed according to Kovats retention index (RI) within each class. * indicates compounds which match the literature's fragmentation spectrum but not by the RI. Chemical Abstracts Service (CAS) registry numbers and literature Kovats retention index (RI) are also provided. Different letters in the same column indicate significant statistic differences (Mann–Whitney U-test; P< 0.05).

Compounds	**CAS**	**RI** (Calculated)	**RI** (Literature)	***D. cogniauxiana***	***D. pendula***	***Pab. jugosa***	***Pro. xanthina***	***W. warreana***	***Z. crinitum***	***Z. maculatum***	***Z. maxillare***
Fatty acid derivatives (%)				-	-	-	-	-	1.11±0.66d	1.17±1.49d	-
Benzenoids (%)				-	99.67±0.36	-	0.22±0.31b	-	25.05±2.06c	-	2.42±4.83d
Terpenoids (%)				91.80±11.20a	-	98.71±0.78a	99.75±0.35a	89.21±6.77a	73.45±2.08a	98.12±2.77a	95.97±4.47a
Monoterpenes (%)				4.19±11.89c	-	0.75±1.05b	98.22±1.46a	-	39.56±4.75b	72.63±9.72b	74.13±10.51b
Sequiterpenes (%)				88.03±15.92b	-	97.97±1.83a	1.53±1.10b	89.21±6.77a	33.90±4.19b	25.49±9.29c	21.84±9.79c
Total N of VOCs detected				10	3	3	6	3	31	8	20
FATTY ACID DERIVATIVES											
3-nonen-1-ol	10340-23-5	1164	1160	-	-	-	-	-	*tr*	-	-
decanal	112-31-2	1213	1213	-	-	-	-	-	*tr*	-	-
2,5-dimethylhexane-2,5-dihydroperoxide	3025-88-5	1364	NA	-	-	-	-	-	*tr*	-	
tetradecane	629-59-4	1403	1400	-	-	-	-	-	-	1.17 ± 1.49	-
dodecanal	112-54-9	1414	1414	-	-	-	-	-	0.28 ± 0.11	-	-
hexadecane	544-76-3	1602	1600	-	-	-	-	-	0.24 ± 0.26	-	-
tetradecanal	124-25-4	1621	1622	-	-	-	-	-	0.50 ± 0.49	-	-
BENZENOIDS											
linalyl anthranilate	7149-26-0	1123	NA	-	-	-	0.22 ± 0.31	-	-	-	-
1,2-dimethoxybenzene	91-16-7	1156	1155	-	-	-	-	-	0.40 ± 0.03	-	-
benzyl acetate	140-11-4	1174	1175	-	-	-	-	-	0.21 ± 0.07	-	-
benzoic acid, 2-hydroxy-methylester	119-36-8	1205	1208	-	-	-	-	-	0.82 ± 0.35	-	-
1,2,3-trimethoxybenzene	634-36-6	1321	1317	-	-	-	-	-	5.40 ± 0.23	-	-

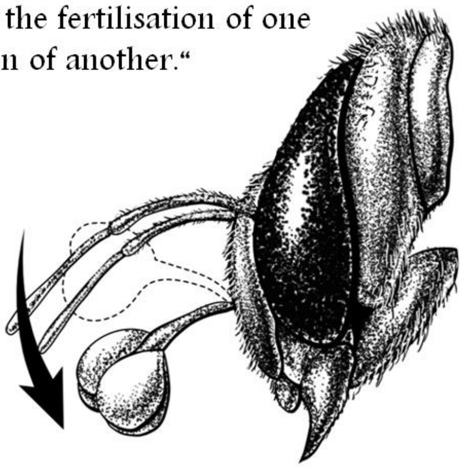
2-methoxy-4-vinylphenol	7786-61-0	1326	1325	-	66.25 ± 57.38	-	-	-	-	-	-
benzoic acid, 2-methoxy-,methylester	606-45-1	1351	1351	-	-	-	-	-	0.26 ± 0.20	-	-
methyl anthranilate	134-20-3	1357	1354	-	-	-	-	-	0.41 ± 0.18	-	-
4-hydroxy-3-methylacetophenone	876-02-8	*	1323	-	33.33 ± 57.74	-	-	-	-	-	-
benzoic acid, butylester	136-60-7	1387	1389	-	-	-	-	-	*tr*	-	-
isoeugenol	97-54-1	1411	1410	-	*tr*	-	-	-	-	-	-
1,2,3,4-tetramethoxybenzene	21450-56-6	*	1533	-	-	-	-	-	17.56 ± 2.05	-	-
2,6-*bis*(1,1-dimethylethyl)-4-(1-oxopropyl)phenol	14035-34-8	1643	1644	-	-	-	-	-	-	-	2.42 ± 4.83
TERPENOIDS – *Monoterpenes*											
β-myrcene	123-35-3	993	993	-	-	-	87.62 ± 8.36	-	0.19 ± 0.05	40.06 ± 5.22	69.01 ± 10.78
s-limonene	5989-54-8	1037	1033	-	-	-	-	-	-	-	*tr*
(z)-β-ocimene	3338-55-4	1043	1043	-	-	-	8.08 ± 5.22	-	1.56 ± 0.26	1.62 ± 0.35	*tr*
(e)-β-ocimene	3779-61-1	1051	1053	-	-	0.75 ± 1.05	2.52 ± 1.68	-	19.59 ± 1.59	30.96 ± 4.51	4.84 ± 0.94
β-linalool	78-70-6	1105	1107	-	-	-	-	-	18.20 ± 2.96	-	*tr*
perillene	539-52-6	1123	1126	-	-	-	-	-	-	-	0.16 ± 0.31
neo-allo-ocimene	7216-56-0	1135	1131	-	-	-	-	-	*tr*	-	-
myrcene disulphide	73188-23-5	*	1555	4.19 ± 11.89	-	-	-	-	-	-	-
TERPENOIDS – *Sesquiterpenes*											
α-longipinene	5989-08-2	1363	1360	-	-	87.37 ± 1.87	-	-	-	-	12.16 ± 4.23
longicyclene	1137-12-8	1388	1392	-	-	-	-	-	-	-	1.73 ± 0.77
β-cubebene	13744-15-5	1392	1392	15.88 ± 6.96	-	-	-	5.51 ± 9.54	-	0.50 ± 0.59	-
farnesane	3891-98-3	*	1379	-	-	-	-	-	0.34 ± 0.52	-	-
bergamotene	55123-21-2	1415	1419	0.31 ± 0.63	-	-	-	-	-	-	-
allo-aromadendrene	25246-27-9	1416	1416	-	-	-	-	-	-	-	1.99 ± 1.18
germacrene d	23986-74-5	1423	1436	0.53 ± 1.60	-	-	-	-	-	-	-
longifolene	475-20-7	1424.3	1423	-	-	-	-	-	-	-	1.40 ± 1.23
(z,e)-α-farnesene	26560-14-5	1475	1478	1.02 ± 2.29	-	-	-	74.57 ± 16.47	1.87 ± 0.53	4.16 ± 1.97	0.28 ± 0.56

caryophyllene	87-44-5	1435	1437	-	-	-	-	-	*tr*	-	0.60 ± 0.69
trans-α-bergamotene	13474-59-4	1442	1446	8.74 ± 7.10	-	-	0.65 ± 0.15	9.13 ± 4.70	*tr*	-	-
α-guaiene	3691-12-1	1451	1452	-	-	-	-	-	*tr*	-	-
α-bergamotene	17699-05-7	1453	1449	-	-	-	0.89 ± 1.25	-	-	-	0.29 ± 0.21
(e)-β-farnesene	28973-97-9	1454	1456	0.34 ± 1.02	-	-	-	-	-	-	-
aromadendrene	109119-91-7	*	1436	-	-	-	-	-	-	-	0.97 ± 1.29
β-farnesene	77129-48-7	1467	1464	60.27 ± 10.17	-	-	-	-	-	-	*tr*
α-himachalene	3853-83-6	1468	1468	-	-	-	-	-	-	-	0.14 ± 0.28
α-zingiberene	495-60-3	1491	1492	0.21 ± 0.62	-	-	-	-	-	-	-
humulene	6753-98-6	1496	1497	-	-	-	-	-	-	-	*tr*
(z)-α-bisabolene	29837-07-8	1497	1498	-	-	10.60 ± 3.70	-	-	-	-	-
α-farnesene	502-61-4	1516	1511	-	-	-	-	-	14.90 ± 0.94	20.77 ± 7.44	*tr*
zingiberene	495-60-3	1523	1521	0.54 ± 1.20	-	-	-	-	-	*tr*	-
calamenene	1460-96-4	1537	1533	-	-	-	-	-	*tr*	-	-
nerolidol	40716-66-3	1544	1544	-	-	-	-	-	*tr*	-	-
(e)-nerolidol	40716-66-3	1572	1572	-	-	-	-	-	0.65 ± 0.45	-	-
farnesyl cyanide	6006-01-5	1587	NA	-	-	-	-	-	13.62 ± 2.02	-	*tr*



"In my examination of Orchids, hardly any fact has so much struck me as the endless diversity of structure ... for gaining the very same end, namely, the fertilisation of one flower by the pollen of another."

(Darwin, 1862)



Chapter II

The dilemma of being a fragrant flower: The major floral volatile attracts pollinators and florivores in the euglossine-pollinated orchid *Dichaea pendula*

Text edited to the journal *Oecologia*

The dilemma of being a fragrant flower: The major floral volatile attracts pollinators and florivores in the euglossine-pollinated orchid *Dichaea pendula*

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In a multidisciplinary and innovative way, this study provides not only basic novel information about the reproductive ecology and pollination mechanism of an orchid, but it presents fresh data on the functional chemical ecology of the interaction between the plant and its florivores, allowing us a mechanistic understanding of two plant-animal interactions: pollination and herbivory.

Author Contributions: CEPN conceived the idea for the study. CEPN and MFGVP performed the experiments and analyzed the data. CEPN, MFGVP, JMB, MJS and MS discussed the results. CEPN wrote the first draft; CEPN, MFGVP, JMB, MJS and MS contributed to the writing of the manuscript.

Abstract

Volatile organic compounds (VOCs) mediate both mutualistic and antagonistic plant-animal interactions, thus the attraction of mutualists and antagonists by floral VOCs constitutes an important trade-off in the evolutionary ecology of angiosperms. Here, we evaluate the role of VOCs in mediating the communication between the plant and their mutualists and antagonists floral visitors. To assess the evolutionary consequences of VOCs mediated signalling to the distinct floral visitors, we studied the reproductive ecology of *Dichaea pendula*, assessing the effects of florivores on fruit set, the pollination efficiency of both pollinators and florivores, the floral scent composition and the attractiveness of the major VOC to pollinators and florivores. The orchid depends entirely on orchid-bees for sexual reproduction, and the major florivores, the weevils, fed on corollas causing self-pollination, triggering abortion of 26.4% of the flowers. Floral scent was composed of *ca.*99% 2-methoxy-4-vinylphenol, an unusual floral VOC attractive to pollinators and florivores. The low fruit set from natural pollination (5.6%) compared to hand cross-pollination (45.5%) and low pollinators' visitation [0.02 visits (flower individual hour)$^{-1}$] evidence pollination limitation.2-methoxy-4-vinylphenol mediates both mutualistic and antagonistic interactions, which could result in contrary evolutionary pressures novo-emission. Scarcity of pollinators, not florivory, was the major constraint to fruit set. Our results suggest that, rather than anti-florivory adaptations, adaptations to enhance pollinator attraction and cross-pollination might be the protagonists in the evolution of VOC emission in euglossine-pollinated flowers.

Keywords: Atlantic Forest, Euglossini, floral headspace, chemical ecology, pollination.

Introduction

Floral volatile organic compounds (VOCs) play an important role in mediating plant-pollinator interactions(Schiestl and Schlüter 2009; Raguso et al. 2015).Together with visual and tactile cues, volatiles are important in both long-distance attraction to and short-distance recognition of floral resources by pollinators(Williams and Whitten 1983; Wright and Schiestl 2009). Floral scent signalling can enhance sexual reproduction in plants by mediating plant-pollinator interactions and thus undergoes natural selection (Schiestl and Johnson 2013; Schiestl 2015). Long-distance attraction by odour cues should be especially important in the tropical forest environment, where most flowers on a plant assemblage are not readily visible to their pollinators. Thus, providing odour pathways to pollinators may be an adaptive strategy for tropical forest plants.

However, other flower foragers, such as florivores, may use the same signals that are used by pollinators to find flowers on which they feed and reproduce(Baldwin et al. 1997; Andrews et al. 2007).The detrimental consequence of providing odour cues that also attract florivores may represent a major trade-off in flower evolution(Baldwin et al. 1997). Furthermore, herbivory activity may negatively affect plant fitness directly via tissue loss and indirectly via changes in VOC emission (Kessler et al. 2011).Thus, flowering plants may have evolved strategies to tolerate and/or deter “unwelcome” flower visitors while attracting pollinators. Such strategies may be related to fragrance emission dynamics as well as to fragrance chemical composition, for example, by emitting herbivore repellents (Schiestl 2015).

The family Orchidaceae comprises huge morpho-functional flower diversity, and a large amount of knowledge of orchid floral volatiles has been accumulated(Kaiser 1993; Knudsen et al. 2006). Thus, orchids constitute an excellent study model to approach relationships among plants, their pollinators and florivores as mediated by floral volatiles(Bronstein et al. 2014). Consequently, *in situ* ecological studies are essential to better understand the actors and roles that are involved in plant-animal interactions mediated by VOCs.

Dichaea Lindl. (Maxillarieae, Zygopetalinae) is a Neotropical genus of epidendroid orchids containing *c.* 120 species of small and mainly epiphytic herbs (Neubig et al. 2009; The Plant List 2010). The published information on pollination in *Dichaea* consists of observations of flower visitors and pollinaria-carrying by orchid-bees (Apidae, Euglossini) in a few Mesoamerican species. The *Dichaea* species that have been studied present similar flower sizes and shapes, fragrant flowers and are visited by male euglossine bees(Folsom 1985; Roubik and Ackerman 1987; Singer and Sazima 2004).

Using *Dichaea pendula* (Aubl.) Cogn. as a study system, we aimed to answer the following questions: What are the pollinators of *D. pendula*, and what adaptations are involved in pollination? How dependent is this orchid species on its pollinators? Is there phenological overlap between the peak of flowering and the richness and abundance of potential pollinators? What are the main volatiles that are involved in pollinator attraction? What are the costs and benefits that are involved in the interaction of this orchid species with pollinators and florivores that are both attracted to the major floral VOC? Upon answering these questions, we expect to reveal the trade-offs between attracting pollinators and attracting florivores that may have been present throughout the evolution of fragrant flowers.

Materials and Methods

Study species and area

Dichaea pendula (Aubl.) Cogn. is found from the lower trunks near the forest soil to the middle canopy as a pendant epiphyte in the Atlantic Forest of south-eastern Brazil (Barros et al. 2014).The lack of pseudobulbs and the presence of single-flowered inflorescences bearing small (less than 3 cm) flowers with anchor-shaped lips are synapomorphies of the genus among the extant Zygopetalinae subtribe (Whitten et al. 2005) (Fig. 1a). Fruits of *D. pendula* are conspicuously spiny, characteristic of the section *Dichaea* of the genus (Neubig et al. 2009) (Fig. 1b). This orchid presents flowers with oval tepals that are primarily coloured cream-white with deep violet spots, and the lip is inversely coloured in deep violet interspaced with cream-white spots (Fig. 1c). The lip bears an appendage at the upper portion of its base close to the stigmatic surface. The viscidium is located directly above (within 1 mm) the stigmatic surface, and the anther cap fully covers the pollinarium except for the viscidium and bears four compressed, round pollinia that are arranged as two superposed pairs (Figs. 1c and 2e).

Field work was carried out between 2012 and 2015 in highland (from 800 to 1000 m a.s.l.) Atlantic forest areas (ombrophilous dense forest, Veloso et al., 1991) in Serra do Mar State Park (SMSP), south-eastern Brazil. The studied populations of *D. pendula* are from two highland areas of secondary (40-50 yrs old) forest between the municipalities of São Luiz do Paraitinga, Cunha and Natividade da Serra, São Paulo State (23º26'08"S-45º13'22.5"W and 23º19'55"S-45 º05'49"W, respectively). Vouchers specimens of the plants (E. de Melo 14-Nov-1998 2555; R.B. Singer 99/38 25-Nov-1999 and C.E.P. Nunes 05 19-Nov-2012) were deposited in the herbarium of the State University of Campinas (UEC).

Breeding system and floral biology

The breeding system of *D. pendula* was assessed using controlled pollination experiments (Radford et al. 1974; Dafni et al. 2005). The pollination experiments included (i) hand cross-pollination (flowers were emasculated and hand-pollinated using one pollinarium from another orchid growing on another tree at least 10 m away), (ii) hand self-pollination (flowers were hand-pollinated using each plant's own pollinarium), (iii) spontaneous self-pollination (unmanipulated flowers were bagged without further treatment), (iv) natural pollination (flowers exposed to pollinators and florivores), and (v) weevil pollination (flowers that were observed to be pollinated by florivore weevils were bagged). The numbers of flowers and individuals in each experiment are presented in Table 1. The time, sequence and duration of anthesis (i.e., the period in which the corolla was open and male or female functional organs were present) were assessed *in situ*. The emission of flower scent throughout the day was also assessed *in situ* by smelling individual flowers or groups of flowers from a distance of 10 cm every 20 min during focal observations.

Pollinators and the mechanism of pollination by pollinarium movement

A total of84.17 h of focal observations of floral visitors was performed during the flowering seasons of 2010/2011, 2012/2013 and 2013/2014from 07:00 to 19:00 h, covering both diurnal and crepuscular periods. During each observation period, one individual bearing 1-10 flowers was observed *in situ* or recorded with a digital camera (DCR-RS68 Sony, Tokyo, Japan). Floral visitors removing and depositing pollinaria were considered to be pollinators. The behaviour of visitors and pollinators were analysed from digital videos and images of focal observations.

In order to assess the abundance-corrected efficiency of pollination by different groups of floral visitors, we calculated the pollination efficiency using the following formula:

$$E = (A \times B^{-1}) \times C^{-1}$$

were E is the relative efficiency of a given pollinator, A is the frequency of flowers pollinated by that pollinator in natural conditions, B is the visitation rate of that given pollinator [(flower individual hour)$^{-1}$] and C is the relative abundance or the fraction of that given pollinator over all the visits to the attractive baits during the field behavioural bioassays. The effectiveness of different pollinators was then evaluated according to the breeding system of the species and by quantifying the fruit set of flowers that were pollinated by distinct visitors (for the concepts of efficiency and effectiveness, see Freitas 2013).

To evaluate the function of pollinaria movement in the pollination and breeding of *D. pendula*, the following field experiment was performed: pollinaria of 19 individual flowers from eight plants were removed, attached to a toothpick supported by a clothespin and laid on a plain surface. The time until pollinarium movement stopped (completed pollinarium reconfiguration) was recorded using a chronometer (click the link https://www.youtube.com/watch?v=AYdoSxqauZ0to watch the pollinarium movement in an example of this experiment).

To determine the ratio of flowers that were lost due to self-pollination by *Montella* sp. n. weevils, all flowers showing evidence of pollination by weevils (i.e., pollinarium twisted downwards and weevil presence on flowers) were tallied during the Dec-2013 field work. The total number of flowers present at that time was estimated by multiplying the mean number of open flowers per individual by the total number of flowering individuals that were recorded in the phenological studies during the same month. This value was then used to calculate the ratio of flowers that were self-pollinated by weevils.

Floral scent analysis (SPME and GC-MS)

Four individual plants of *D. pendula* bearing flower buds were collected in the field, transferred to a greenhouse at the State University of Campinas (Unicamp) and mounted on giant fern (*Dicksonia* sp.) fibre plaques during the 2012/2013 flowering season. As the flowers opened, flower-bearing ramets were bagged with polyester bags (27 × 41 cm) and left for one to three hours to build up floral VOCs and reach flower-air equilibrium. Thereafter, the bags were perforated with a pin and exposed to a solid phase micro-extraction (SPME) syringe carrying a polydimethylsiloxane fibre (PDMS, 100 µm, Supelco, Bellefonte, PA) for 15 minutes. Collections were performed from 10:00 to13:00 h on sunny days at 20-30 ºC, as *D. pendula* fragrance was most often detected by the human nose at these times and under these conditions in the field.

Immediately after collection, SPME fibre samples were directly injected into a gas chromatograph(2010A, Shimadzu, Tokyo, Japan) that was coupled to a quadrupole mass spectrometer (QP2010, Shimadzu) using a DB5 capillary column (30 m length, 0.32 mm internal diameter and 0.25 µm film thickness, J&W Scientific, Folsom, CA, USA) and helium as a carrier gas (flow of 1 mL.min^{-1}). Injection was performed in splitless mode, and the fibre was held for 20 min in the injector at 200 ºC with the transfer line at 240 ºC to elute VOCs. The oven temperature started at 50 ºC and then increased by 10 ºC min^{-1} to a maximum temperature of 250 ºC and was then held for 10 min until the end of the run (Cancino & Damon, 2007). Mass spectra were recorded by electron impact (EI) at 70eV using the SIM mode. We calculated the Kovats Retention Index (RI) of each compound peak from a previously injected, homologous series of n-alkanes (C8-C20). Compound peaks were identified by comparison of the mass spectra and RIs to those of the NIST05 and NIST online library (Linstrom and Mallard 2011) and *The Pherobase* semiochemical database (El-Sayed

2012). In the case of 2-methoxy-4-vinylphenol, the mass spectra and retention time were compared with those of the analytical standard (Sigma-Aldrich, Saint Louis, IL, USA).

Behavioural bioassays

Dual-choice bioassays were conducted *in situ* to test the attractiveness of the dominant fragrance component 2M4VP to floral visitors of *D. pendula* in Jan-2015 and Feb-2015.These assays were performed from 08:00 to 15:00h on five sunny days. Each replicate consisted of a pair of circular filter-paper baits (Whatman #1; 11 cm diameter) that were hung by a cotton line in the vegetation of the forest border or along forest paths (Fig. 3b, arrows) at a distance of 1 m within a pair and at least 3 m (3-30 m) between different pairs. In each pair, 0.5 ml of 2M4VP pure analytical standard was applied to one piece of paper, and nothing was applied to the other (control). Three to five assays were performed simultaneously on the same day, yielding a total of 19 replicates. A choice was recorded each time an insect landed and then remained for at least 5 s on either the bait or vegetation within a 10-cm radius of the bait. All of the insect visitors to the baits were recorded, and all of the coleopterans were collected alive for identification and posterior tests in the laboratory. Orchid-bee visitors were identified and recorded but not collected.

In Jan-2015, to test whether *Montella* sp. n. were attracted to2M4VP and to validate the field results, dual-choice bioassays were also performed under more controlled conditions at the Laboratory of Chemical Ecology and Insect Behaviour at "Luiz de Queiroz" College of Agriculture (EALQ). Adult *Montella* sp. n. that had been collected from *D. pendula* and *D. cogniauxiana* plants as well as from baits of the previous field bioassays were maintained for one to three weeks in flasks containing 10% honey solution. The choices of these weevils were assessed in glass Y-tube olfactometers (10.0 cm arm length and 1.5 cm internal diameter), with 2M4VP and solvent as a control offered as stimuli. The terminals of the olfactometer side arms were connected to a Volatile Collection System (ARS, Gainesville, FL, USA), which regulated the clean air flow at a rate of 0.4 $L.min^{-1}$ per arm. For each replicate, a single weevil was introduced in the main arm of the Y-tube. A choice was recorded when the insect crossed a line located 3 cm from the terminal of one of the side arms and remained there for at least 5 s. If the weevil did not respond (i.e., did not cross the line of one of the side arms within 20 min), the insect was considered non-responsive and was not included in the statistical analysis. A total of 28 weevils were used, yielding 20 choices. Weevils were tested only once, and for each replicate, a pair of rubber septa that were impregnated with either 10 µL of 2M4VPat 10 $ng.\mu L^{-1}$ or hexane (control) was used as odour sources that were placed at the terminals of the side arms. The Y-tube olfactometers were washed with hexane and acetone and dried at room temperature for 10 min between trials. Bioassays were conducted in an illuminated room that was maintained at 26.0 ± 0.5 °C from 9:00 to 15:00 h on sunny days. Choice proportions were analysed by the exact binomial test of goodness-of-fit using the R statistical environment (R Development Core Team, 2013).

Flowering, odour emission and euglossine bee temporal activity patterns

To test whether flowering and odour-signalling activities coincided with pollinator occurrence and odour-collecting activity, respectively, flowering activity was recorded monthly from Sep-2011 to Aug-2014 (Newstrom et al.1994). In addition, male orchid-bees (Hymenoptera, Apidae, Euglossini) that were attracted to the chemical baits were recorded and/or collected from Mar-2012 to Aug-2013 between 9:00and 14:00 h. The phenological stage (flowering or vegetative) of each *D. pendula* plant along at least 12 km of trails throughout the forest was recorded. Only *D. pendula* plants presenting evidence of previous/current flowering (e.g., fruits, dry fruits, or dry flower axes) were included in this study(210.8 ± 84.9 plants per month, mean ± SD, *n* = 36 months).In contrast to the other assays of the study, we used 1,8-cineol, methyl salicylate, benzyl acetate, eugenol and vanillin to attract male orchid-bees, as these are known attractants of a wide array of orchid-bee species in the study site (Singer and Sazima 2004). In addition, the monthly abundance and richness of euglossine potential pollinators were estimated, and the time interval of odour emission was recorded from one flower from eight different plants in the 2010/2011 and 2011/2012 flowering seasons. To determine daily bee activity, only data from November to January were used, corresponding to the flowering period of *D. pendula* in the study area.

The null model approach (Castro-Arellano et al. 2010) was used to test the overlap between both annual and daily activity patterns of flowering and euglossine bee activity. Null models with a random overlap between bee- and plant-activity patterns were created using the ROSARIO algorithm of the Time Overlap software (Castro-Arellano et al. 2010). The Czechanowski (IC) and Pianka (IP) indexes of temporal niche overlap were calculated for our data and for each of the 10,000 iterations of the null model. The probabilities were then calculated by testing the null hypothesis that the observed temporal niche overlap index was greater than that expected by chance.

Results

Floral biology and breeding system

Dichaea pendula produced 5.5 ± 5.4 flowers per plant per flowering season (mean ± SD, *n* = 118) and bore 2.3 ± 1.7 (mean ± SD, *n* = 111) open flowers per plant from November to January, the flowering season. Flowers lasted *ca.* 2 days, opening in the morning and partially closing in the afternoon (after *ca.* 15:00 h). On the afternoon of the second day, flowers fully closed. From late morning (10:00h) until the beginning of afternoon (13:30h), flowers emitted a conspicuous odour resembling that of clove oil or eugenol. The studied populations of *D. pendula* were completely self-incompatible and pollinator-dependent, producing no fruit from hand self-pollination, autonomous self-pollination or weevil pollination, with 45.45% of the fruit set resulting from hand cross-pollination and 5.6% of the fruit set resulting from natural pollination (Table 1). Fruit set from hand cross-pollination approximated a 50% ratio (exact binomial test, h_0: fruit set ratio$\neq$ 0.5; $p = 0.73$).

Pollinators and pollination mechanism by pollinarium movement

The flowers of *D. pendula* were visited by male orchid-bees of *Eufriesea violacea* and *Euglossa* sp. at a rate of 0.02 visits (flower individual hour)$^{-1}$, and weevils *Montella* sp. n. at a rate of 0.04 visits (flower individual hour)$^{-1}$(Fig. 2).

Males of the orchid-bees *Eufriesea violacea* and *Euglossa* sp. visited flowers and removed pollinaria of *D. pendula*. One *Eufriesea violacea* visited a 10-flower plant and removed pollinaria, which attached near the top of the head, from three flowers (Fig. 2a-c), but it deposited no pollinaria. *Eufriesea violacea* spent an average of 15.00 ± 4.08 s (mean ± SD, $n = 3$) collecting odour from each flower of the 10-flowered plant, whereas an individual *Euglossa* sp. was observed spending five s collecting from a single-flower plant.

Male orchid-bees visited *D. pendula* flowers and collected odour from the lip in a specific manner. After landing on the lip, they grabbed the lip appendage with their mandibles and scraped the lip surface with their anterior and median legs. During this process, the bee contacted the viscidium such that the pollinarium attached near the top of its head (Fig. 2d-e). Soon after removal, pollinia were positioned farther upward on the head, preventing their deposition on the stigma (Fig. 2e). Only after the supination movement of the caudicle, which moves the pollinia downward, were the pollinia susceptible to deposition on the stigma. The complete pollinaria movement lasted an average of 190.89 ± 108.86 s (mean ± SD, $n = 19$), which was longer than the mean visiting time of orchid-bees at individual flowers, which was 28.10 ± 12.56s (mean ± SD, $n = 4$) (one-tailed Student's t-test, $p < 0.001$).

Montella sp. n. individuals spent from five minutes to several hours on flowers, feeding on the corolla, the anthers' caudicle and viscidium without totally destroying the flowers or causing any perceptible decrease in corolla area. Furthermore, weevils mated on flowers, eventually transporting pollinaria with their rostrum and forelegs, resulting in self-pollination (click the link https://www.youtube.com/watch?v=QkkEjWvc2o4 to watch a video of the feeding/pollinating behaviour of a *Montella* sp. n. on *D. pendula* flower). However, weevils did not carry pollinaria on their bodies or transport them between different flowers (Fig.2f-h).Orchid-bees showed a relative efficiency of 133.33, 20 times higher than the relative pollination efficiency of weevils, which was of 6.63. During the Dec-2013 field work, a total of 22flowers (26.43% of the estimated total) were recorded as self-pollinated by weevils and none of them resulted in fruit (Table1).

Floral scent analysis

Three of the four sampled flowering plants provided detectable amounts of VOCs. Two of these plants emitted blends with 2M4VPas the major compound (99% of the total amount) and isoeugenol as a minor compound (Fig. 3a). These two samples corresponded to plants with flowers that released a human-detectable smell of clove at the time of sampling. The third sample did not present any nose-detectable smell and was composed only of trace amounts of 4-hydroxy-3-methylacetophenone. Thus, *D. pendula* flower scent is predominantly composed of 2M4VP and may present traces of isoeugenol and 4-hydroxy-3-methylacetophenone.

Behavioural bioassays

In field bioassays, pure 2M4VP attracted males of the orchid-bee species *Eufriesea violacea* (two individuals), *Euglossa* sp. (one individual) and *Eulaema marcii* (one individual), as well as 228 *Montella* sp. n. weevils (exact binomial test, $p < 0.001$, Fig. 4). During visits to the baits, male orchid-bees landed on the filter paper and actively collected2M4VP in their typical fragrance-collecting behaviour (Fig. 3c-d). By contrast, *Montella* sp.n. weevils landed on the filter paper or nearby vegetation and walked towards the fragrance source, stopping when they reached the source or standing still without any perceptible action except for antenna and head movements. The florivore *Montella* sp. n. weevils were attracted to the chemical baits at a rate of 46:1in relation to the orchid-bee pollinators. Y-tube olfactometer assays confirmed the attraction of *Montella* sp. n. weevils to 2M4VP (exact binomial test, $p = 0.04$, Fig. 4).

Flowering, odour emission and euglossine bee temporal activity patterns

Dichaea pendula exhibited an annual flowering pattern: flowers were present during the warmest/rainiest season from November to January, with a peak in flowering between November and December (Fig. 5a). Adult male orchid-bees were active at the study sites for most of the year, but they were not detected in June or July, the period with the lowest temperatures. A total of 35 individuals of seven species belonging to the genera *Eufriesea* (one individual), *Euglossa* (21 individuals of four species) and *Eulaema* (13 individuals of two species) were recorded on the chemical baits that were used to determine the year-round activity of orchid-bees. No bees were recorded on the chemical baits in June or July. *Euglossa* bees were present in all months except for Aug-2012, and *Eulaema* bees were absent only in Sep-2012. *Eufriesea* was the rarest bee genus, with a single individual recorded in Dec-2012. The abundance and richness of orchid-bees exhibited the greatest peaks in the middle of the warmest and rainiest season (Dec-2012) and the weakest peaks at the end of the warmest/rainiest period of the year, in Mar-2012 and Mar-2013 (Fig. 5a).

Flowering peaks of *D. pendula* occurred within the period of odour-collecting activity of its male orchid-bee pollinators, August to April, which was the period of the year with the highest temperature in the study area. However, the plant's flowering peaks did not coincide significantly with the peak of either abundance (IP = 0; IC = $1.5E^{-11}$; $p_{(IP \geq observed)} = 1.00$; $p_{(IC \geq observed)} = 0.99$) or richness of orchid-bees (IP = 0; IC = $1.5E^{-11}$; $p_{(IP \geq observed)} = 1.00$; $p_{(IC \geq observed)} = 0.99$).The flowers began to release odours between *ca.* 09:30 and 10:00h, the period when Euglossini pollinators were already actively collecting odours, and ended between 13:00 and 14:00 h, the period when pollinators were no longer collecting odours (Fig. 5b). Again, there was no significant overlap between flower odour emission and pollinator odour-collecting activities (IP = 0.49; IC = -2.56; $p_{(IP \geq observed)} = 0.57$; $p_{(IC \geq observed)} = 0.59$).

Discussion

Breeding system and floral biology

Unlike most orchid species, including other euglossine-pollinated sympatric species (Neiland and Wilcock 1998; Pansarin et al. 2006; Pansarin and Amaral 2009), *D. pendula* is completely self-incompatible. This trait is advantageous when efficient pollinators and pollination mechanisms are present, as they provide sufficient pollination service and prevent inbreeding, respectively. Nevertheless, the observed low fruit set under natural conditions relative to the hand cross-pollination treatments (Table 1) together with low visitation frequencies indicate that pollination is limited because of a scarcity of pollinators. Other systems involving tropical Orchidaceae, including several euglossine-pollinated taxa and other angiosperms show that pollination limitation may be the rule for the Atlantic forest ecosystems and for orchids (Tremblay et al. 2005; Pansarin et al. 2006; Pansarin and Amaral 2009; Vale et al. 2011; Wolowski et al. 2014).

The crossing/pollinator-dependent breeding system of *D. pendula* likely promotes reduced fitness in the presence of pollinators that effect self-pollination and consequently lead to flower loss. However, according to the geographic mosaic theory of coevolution (Thompson 2005; Thompson and Fernandez 2006), we expect that the outcomes of the interactions between plants and pollinators or antagonists may vary in time and space. The hypothesis that *D. pendula* populations elsewhere and/or other *Dichaea* species may have self-compatible or more flexible breeding systems that benefit from the visits of florivore weevils is a plausible possibility that is supported by studies of other Orchidaceae (Gamisch et al. 2014). Moreover, results of a study on the pollination system of *Dichaea cogniauxiana* (C.E.P. Nunes, unpublished data) and a study of *Grobya amherstiae* (Catasetinae; Mickeliunas et al., 2006) indicate that some self-compatible orchids benefit from self-pollination by weevils.

Pollinators and pollination mechanism by pollinarium movement

Orchid-bees are the main pollinators of orchids of the subtribes Catasetinae, Stanhopeinae and Zygopetalinae (Vogel 1966; Dressler 1968; Ramírez et al. 2011) and other Neotropical plant taxa (Rocha-Filho et al. 2012; Pérez-Barrales et al. 2013). The importance of male orchid-bees as pollinators is attributed to their trap-lining odour-collecting behaviour and long-distance flying ability (Roubik and Ackerman 1987; Eltz et al. 2005; Eltz et al. 2006).In contrast, *Montella* weevils, as well as other species of Baridinae, are known orchid florivores(Lima 1936; Prena 2008), but little of their natural history has been documented. The evolutionary history of the Curculionidae is filled with cases of the specialization of adult behaviour and larval habits exploring a wide diversity of host plants(Marvaldi et al. 2002). Therefore, we expected *Montella* adults and larvae to be adapted to live and feed on orchid flowers and/or fruits as well as to provide pollination service to self-compatible orchids. Nevertheless, our results evidenced a direct impact of *Montella* weevils on *D. pendula* fitness resulting from flower loss (florivory) and self-pollination and we do not exclude the hypothesis that damage by *Montella* weevils on *D. pendula* flowers causes indirect effects on plant-insect interactions, resulting from changes in the volatile profile of flowers or vegetative parts, as observed by Kessler et al. (2011) in another system. Despite being 46 times less abundant, orchid-bees were 20 times more efficient in pollinating flowers of *D. pendula* than the weevils. That may result from the higher body mass and flight ability, longer flight distances and traplining behaviour of orchid-bees in relation to *Montella* sp. weevils (Ohashi and Thomson 2009).

Pollinarium movement and its consequent reconfiguration (Fig. 2e) spans a longer period of time than the mean duration of a visit by orchid-bee pollinators to *D. pendula* individuals. This information together with the observations that this orchid species produced few flowers in a season (5.46 ± 5.36) and that a low number of flowers are open simultaneously (2.25 ± 1.73) suggest that this process may be the mechanism promoting cross pollination and avoiding geitonogamy. The low number of flowers open simultaneously assures that pollinia from a particular individual will not be deposited in the same individual or another nearby individual that has a high probability of close genetic relationship (Trapnell et al. 2013). The mechanics and physiology of this movement have yet to be studied in detail and maybe similar to the pollinarium movement/reconfiguration that is found in other Epidendroideae orchids (Johnson and Edwards 2000; Peter and Johnson 2009). Furthermore, other *Dichaea* species with different floral traits show different pollination strategies (C.E.P. Nunes, unpublished data).

Ecological role of the major floral volatile 2M4VP and other floral VOCs

The major component of *D. pendula* fragrance,2M4VP, is an unusual component of floral scent (Knudsen et al. 2006). This compound has been identified mainly in essential oils and other tissue extracts of plants belonging to a diverse range of taxa (Linstrom and Mallard 2011). For example, it is a component of buckwheat seeds (*Fagopyrum esculentum*, Polygonaceae; Janeš et al., 2009) and coconut palm stems (*Cocos nucifera,* Arecaceae).In the latter, 2M4VP acts as a kairomone, attracting the stem-drilling weevil *Rhynchophorus ferrugineus* (Coleoptera, Curculionidae) to palms (Gunawardena et al. 1998).

In the present study system, 2M4VP emitted by *D. pendula* flowers is of great ecological relevance as it apparently mediates both beneficial and detrimental interactions with Euglossini pollinators and the florivore *Montella* sp. n. The emission of 2M4VP might have been modulated by these two opposing selective forces in a similar way to the emission of benzyl acetone by flowers of *Nicotiana attenuata* (Baldwin et al. 1997).The weevil might have evolved the adaptive strategy of eavesdropping communication between *D. pendula* and its Euglossini pollinators. Meanwhile, *D. pendula* may have evolved the strategy of emitting the minimum amount of 2M4VP to attract pollinators, preventing the excessive attraction of florivores and conserving resources (Wright and Schiestl 2009), as this epiphytic orchid may be resource limited (Meléndez-Ackerman et al. 2000). Another hypothesis to explain the attraction of *Montella* sp. n. weevils to 2M4VP is a pre-existing sensorial bias of attraction of the weevils to this VOC, which could be a compound previously used in the intraspecific communication of *Montella* sp. n., as observed by Schiestl & Dötterl (2012) in the evolution of other plant-coleopterans systems. The later hypothesis is supported by the fact that other Curculionidae also respond to 2M4VP (Gunawardena et al. 1998).

Furthermore, the floral VOC blends of this species may be composed by pollinator-attracting but also by herbivore-avoiding VOCs, as the result of selective pressures of both pollinators and generalist herbivores (Andrews et al. 2007; Theis and Adler 2012; Kessler et al. 2013). As demonstrated by Kessler et al. (2013) for *Petunia* flowers, the isoeugenol, found here in trace amounts in the floral scent of *D. pendula*, could act as an herbivore deterrent. This way, the orchid-specialized herbivore weevils *Montella* sp. would have evolved strategies not only to track orchid flowers scents but also to deal with orchid chemical defences.

Flowering and euglossine bee temporal activity patterns

The overlap of *D. pendula* flowering and pollinator activity (Fig. 5) is an essential trait for this self-incompatible and pollinator-dependent orchid. Advantages of synchronizing the flowering/anthesis period with pollinator activity periods both throughout the year and the day have been shown in orchids(Sletvold et al. 2010).The existence of more than one peak of richness and abundance of orchid-bees throughout the year as reported here (Fig. 5a) is consistent with the data of Roubik & Ackerman (1987) in Mesoamerica, Ramírez et al. (2015) in Colombia and Singer & Sazima (2004) at a location near the present study area, yielding a global pattern of multiple annual peaks of abundance and richness of orchid-bees. Such a pattern may result from the seasonality of meteorological conditions that determine the abundance of food flowers for the bees, generating pulses of adult emergence and a regular turnover of orchid-bee populations (Roubik and Ackerman 1987).

It remains unclear whether the *D. pendula* phenology and flower odour-emission daily pattern evolved to optimize pollination success through the coinciding of floral scent emission with pollinator occurrence and richness peaks. Alternatively, timing floral emission by *D. pendula* (Fig. 5b) could be a strategy for escaping the florivore abundance peak or may reflect other factors, such as constraints in plant growth and development. As there was no significant overlap between flowering and pollinators activities, our results support the latter two hypotheses.

The annual single-peak pattern of *D. pendula* flowering (Fig. 5a) may be an adaptation to the most effective pollinator; i.e., the flower visitor and species most abundant on 2M4VP baits, *Eufriesea violacea.* The observations that *Eufriesea* bees also have an annual single-peak pattern (Roubik and Ackerman 1987; Singer and Sazima 2004) and that we recorded *Eufriesea* sp. only in December 2012, in the middle of the warmest season, support this hypothesis.

Conclusion

To attract its pollinators, *D. pendula* relies mainly on the production of a single floral volatile, 2M4VP, an attractant to male euglossine bees. However, 2M4VP also attracts the main florivores of *D. pendula*, weevils *Montella* sp. n., which feed on floral parts and inefficiently self-pollinate the flowers, providing no apparent benefit to the plant. Hence, two contrary selective pressures are expected to be mediated by 2M4VP: the positive cross-pollination by euglossine bees and the negative florivory and self-pollination by weevils. Thereby, we expect *D. pendula* flowers to have adaptations to address such conflicting demands. Such adaptations may be related to floral biology, in particular phenology, flower longevity and fragrance emission timing. In fact, we observed flowering phenology to be synchronized, but not fully overlapped, with the occurrence and activity of pollinators. Nevertheless, florivory does not seem to be the major constraint to sexual reproduction in *D. pendula*. Given that pollination limitation is widespread among orchids, more so than anti-florivory adaptations, we expect that adaptations to enhance pollinator attraction and cross-pollination are the protagonists in the evolution of euglossine-pollinated orchids. We believe that the study system involving *D. pendula*, florivores and pollinators composes a good model to better understand the role of floral volatiles in mediating plant-antagonist-mutualist interactions due to its simplicity and manipulability.

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Tables

Table 1. Fruit set, number of fruits and total number of flowers (fruits/total) and number of plants(*n*) that were used in controlled pollination experiments that were performed in a population of *ca.* 210 individuals of *Dichaea pendula* in the Atlantic forest of south-eastern Brazil.

experiment	fruit set %	fruits/total	*n*
hand cross-pollination	45.45	15/33	18
hand self-pollination	0	0/24	16
spontaneous self-pollination	0	0/32	7
natural pollination	5.6	7/125	26
weevil pollination	0	0/25	23

Figures

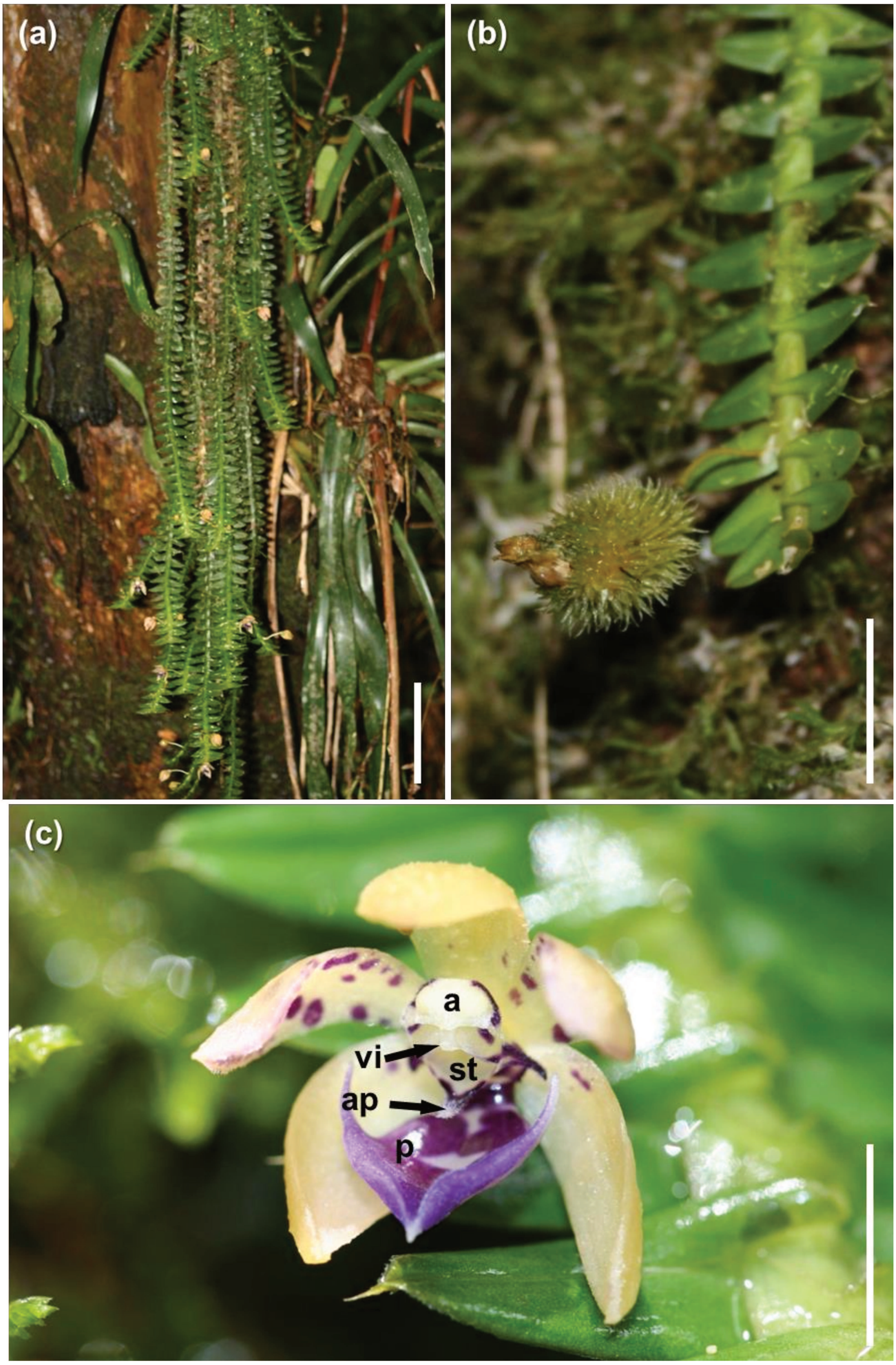

Fig. 1 Plant habit, fruit and flower of *Dichaea pendula*. (a) Flowering plant growing with the typical pendant habit; scale bar = 12 cm. (b) Ramet bearing the conspicuous spiny fruit; scale bar = 1.5 cm. (c) Morphological details of the flower, with the anchor-shaped lip bearing a trichomatous appendage at the adaxial part of its base (ap); scale bar = 1 cm. a = anther cap; vi = viscidium; st = stigmatic cavity; p = odour-containing surface.

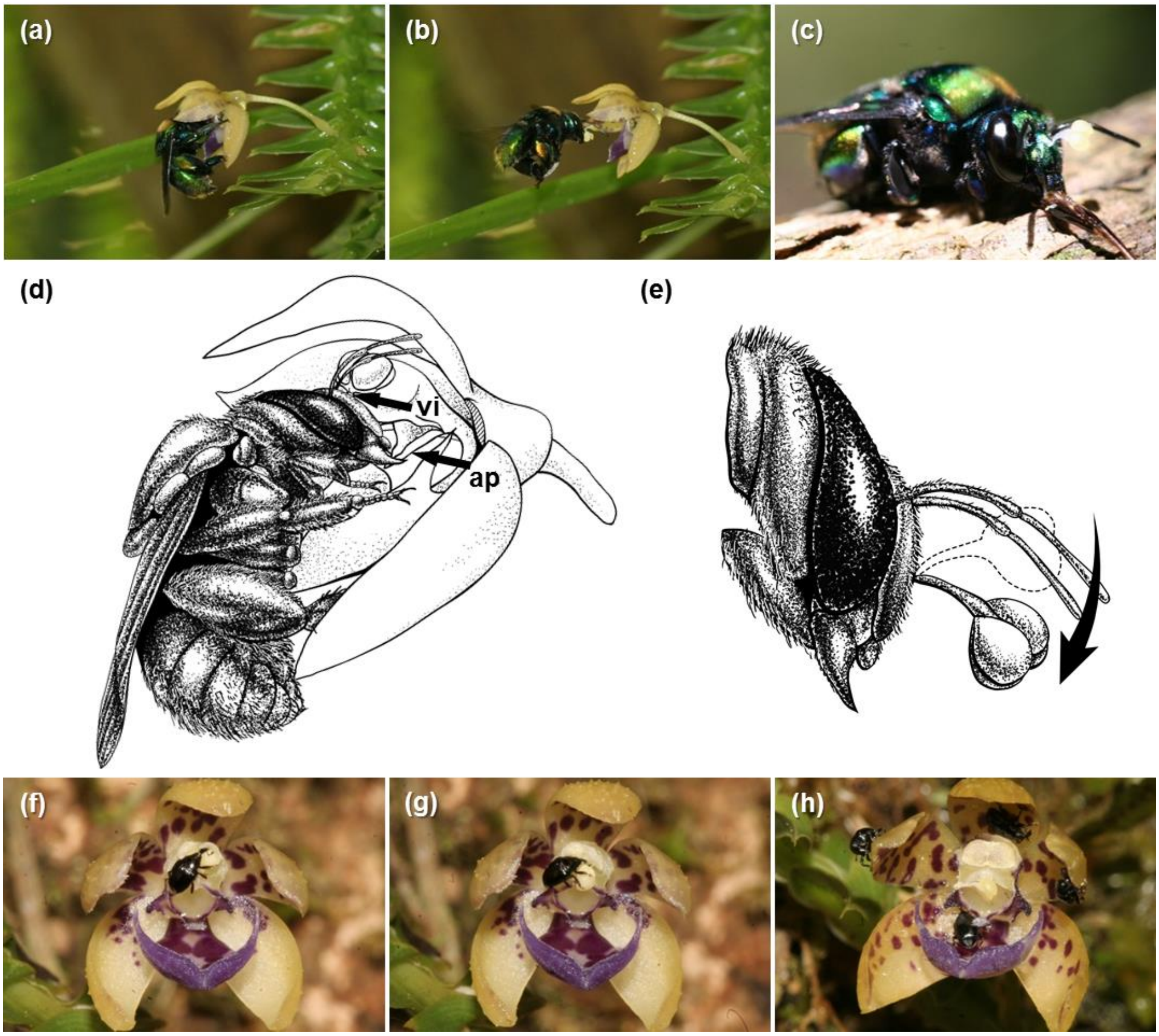

Fig. 2 Pollinator and florivores of *Dichaea pendula*. (a) Male *Eufriesea violacea* on *D. pendula* flower, collecting odour from the lip. (b) The same individual bee leaving the flower with two pollinaria attached near the top of its head. (c) *Eufriesea violacea* with a pollinarium recently attached to its head; note the upward position of the pollinarium. (d) Details of *E. violacea* collecting odour while grabbing the appendage of the lip (ap) and touching the viscidium region (vi) of *D. pendula.* (e) Detail of the bee's head showing the pollinarium movement (arrow): initial (dashed lines) and final position. (f) *Montella* sp. nova initiating the removal of pollinaria from the anther region. (g) *Montella* sp. n. exposing the pollinarium after removing it from underneath the anther cap. (h) Four *Montella* sp. n. in a flower after self-pollination by weevils.

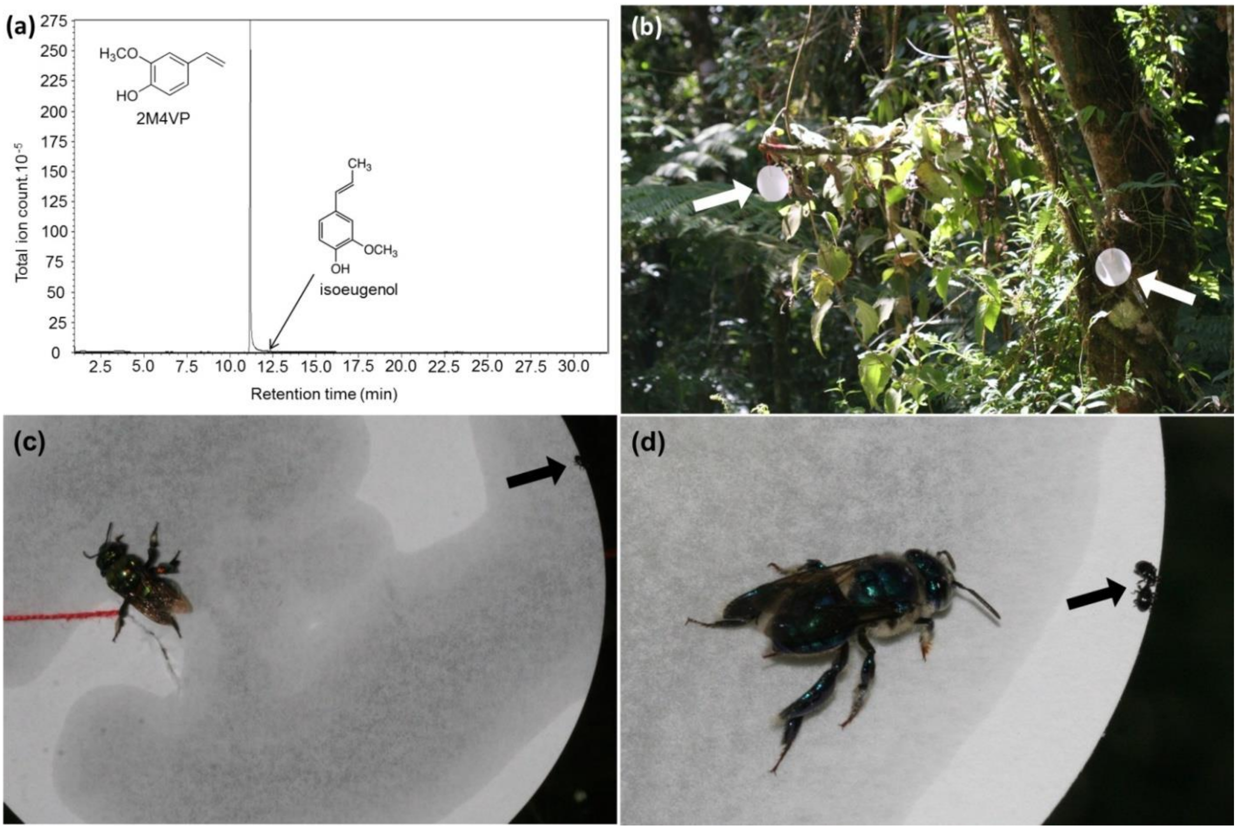

Fig. 3 Total ion chromatogram of *Dichaea pendula* flower emission, potential pollinators and florivores at 2-methoxy-4-vinylphenol (2M4VP) baits. (a) Chromatogram showing the major peak (99.47% of total area) of 2M4VP and a minor peak (0.24%) of isoeugenol, with the respective chemical formulas. (b) Lure and control baits (arrows) hanging on vegetation in a field bioassay. (c) Male euglossine orchid-bee *Eufriesea violacea* collecting 2M4VP from the chemical bait accompanied by a *Montella* sp. nova weevil (arrow). (d) Male euglossine orchid-bee *Euglossa* sp. collecting 2M4VP from the chemical bait accompanied by two *Montella* sp. n. weevils (arrow).

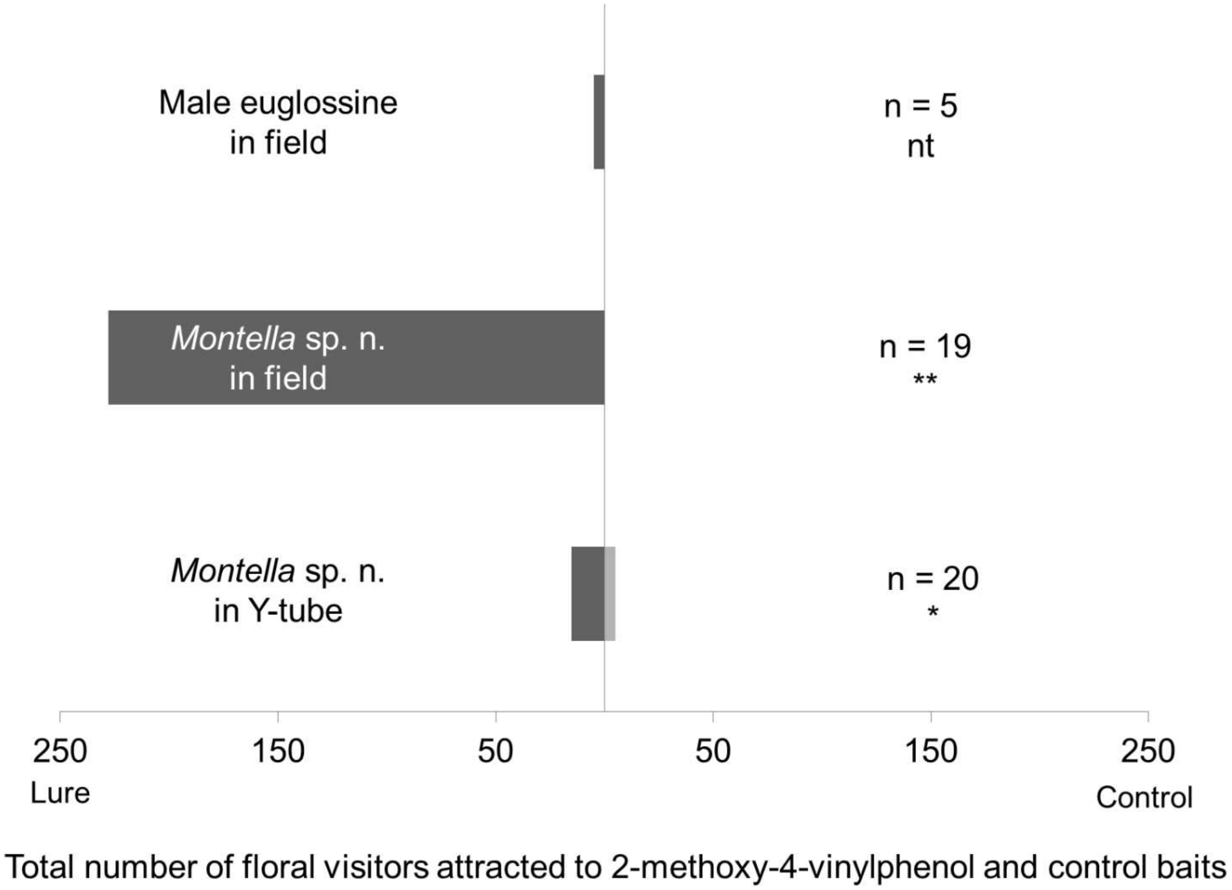

Fig. 4 Behavioural response of male orchid-bees and weevils *Montella* sp. nova to 2-methoxy-4-vinylphenol (2M4VP) in field and laboratory dual-choice bioassays. In the laboratory, weevil preference was measured based on the choice of side arm in a Y-tube, with each arm containing a rubber septum incorporated with either a 10 µL solution of 2M4VP at 10 ng.µL^{-1} or control (10 µL of hexane). In field bioassays, baits were filter-paper disks that were incorporated with 500 µL of undiluted synthetic 2M4VP, whereas controls were filter-paper disks with no substances added. The male orchid-bees included *Eufriesea violacea*, the pollinator of *Dichaea pendula*; *Euglossa* sp. and *Eulaema marcii*, the latter of which was not observed on flowers. Exact binomial tests were performed (lure *vs.* control) where applicable: * $p \leq 0.05$; ** $p \leq 0.001$; nt: not tested because of an insufficient number of attracted bees.

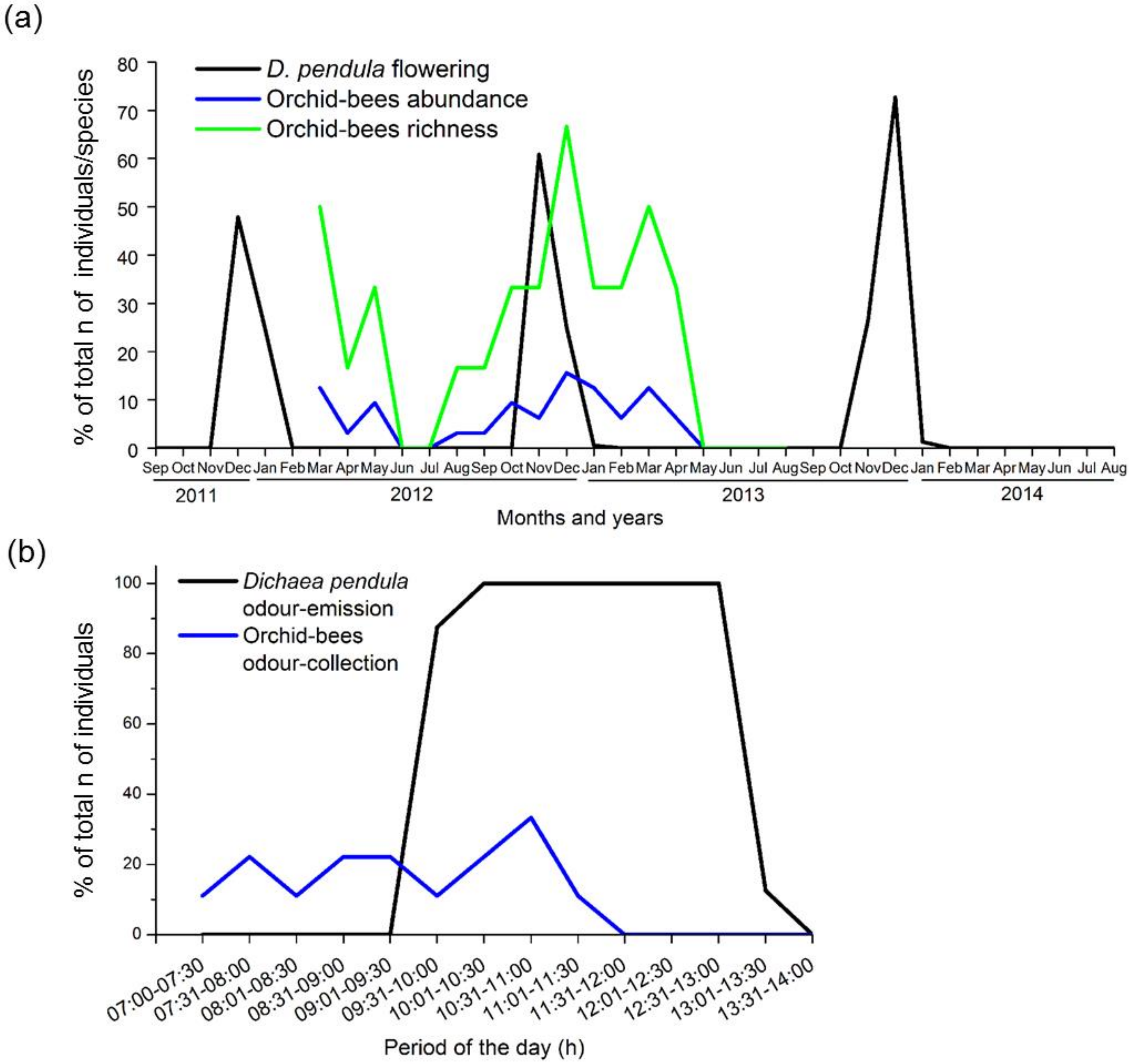

Fig. 5 Year-round and daily floral activity of *Dichaea pendula* and odour-collecting activity of euglossine orchid-bees. (a) Flowering phenology (% of flowering individuals), richness and abundance of orchid-bees pollinators (% of the total number of individuals and species, respectively). (b) Flower odour-emission throughout the day of eight flowers from different individuals on eight different days.

“A aranha tece puxando o fio da teia
A ciência da abeia, da aranha e a minha

Muita gente desconhece
Muita gente desconhece, olará, viu?
Muita gente desconhece”

(João do Vale)

Chapter III

Can orchids be deceived by weevil pollinators? A novel nursery pollination system provides insights on the evolution of pollination from herbivory

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Can orchids be deceived by weevil pollinators? A novel nursery pollination system provides insights on the evolution of pollination from herbivory

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Author Contributions

C.E.P. Nunes performed all the field work and, together with M. Sazima, conceived the manuscript. D.C. Bená and S. Vanin identified the specimens and characterized the larval stages of the life cycle of the *Montella* sp. n. weevils.

Summary

Nursery pollination systems are mutualistic systems in which insects pollinate flowers and lay eggs in floral parts or developing ovules which will feed the larvae so the plant resource to pollinators is generally a portion of the plant's offspring[1–3]. Despite the rarity among flowering plants[1], nursery pollination by fig wasps is widespread in the pantropical fig trees[4]. Such systems are excellent models for studying plant-pollinator interactions because both plant and pollinator fitness can be assessed in the same currency: fruit and seed yields[5]. We investigate an intriguing novel nursery pollination system involving the pollination of orchids by florivore weevils. Female weevils of *Montella* sp. nova actively pollinate flowers of *Dichaea cogniauxiana* and oviposit in flowers, ritualistically repeating this behaviour in each unpollinated flower. Weevil's larvae feed on the developing ovules causing complete loss and abortion of infested fruits. Yearly, 22 ± 6% of the fruits set under natural conditions are infested by weevils. Thus, non-nursery pollination and/or egg-larva mortality are likely to occur. Although 79 ± 11 % of the larva-containing fruits are lost, 18 ± 7% are "rescued" by parasitoid wasps (Braconidae), which kill weevil's larva before extensive damage is inflicted in fruits, which, in this case, proceed to dehiscence and seed release. The weevil-parasitoid temporal dynamics varies spatially in plant patches, but it overall behaves like a predator-prey interaction. More than one species of *Montella* weevils interact with at least ten sympatric orchid species more or less phylogenetically close to *Dichaea* in different ways involving simple florivory of unpollinated flowers by weevil larvae and possibly other cases of nursery pollination[6]. The orchid-weevil system is an excellent model to understand the evolution of pollination mutualisms from florivory, allowing us to approach this problem from autoecology to community ecology levels of plant-animal interactions.

Introduction

Nursery pollination systems are mutualistic interactions in which an insect pollinates flowers and lay eggs in floral parts or developing ovules which will feed the larva while fruit develops, so the resource offered by plants to pollinators is generally a portion of the plant's offspring[1–3]. Despite the rarity among flowering plants[1], nursery pollination by Agaonid fig wasps is widespread in the pantropical fig trees (*Ficus* spp.)[4]. Thereby, in such systems, increase in the pollinator fitness may directly result in a decrease in the plant fitness, and traits that avoid overexploitation of plants by pollinators, necessary to the stability of these systems may have been positively selected[7].

In the context of the coevolutionary theory, we expect that the outcome of a given interaction varies for their participants from negative to positive in time and space and among individuals[8–10]. Additionally, theory predicts that the individual, population and species traits affect not only the possibility of this interaction to occur but also the outcome of the interaction to the individuals involved[11–14]. In the specific case of plant-pollinator interactions, including nursery pollination, that assumption may also be applied and it is possible of testing[8,15]. More specifically, the theory of the geographic mosaic of coevolution predicts that the outcome for the participants in a plant-pollinator interaction may vary in time and among different populations along the geographic distribution of the involved organisms. Therefore, a given plant-pollinator interaction may vary from mutualistic to antagonistic depending on time and space; however the net outcome shall be positive to both involved organisms so the interaction is preserved across time, constituting an evolutionary stable strategy. In nursery pollination systems, we expect that factors inherent to plants (thereby variable among plant individuals), such as selective abortion of over-infested fruits or attraction of herbivore antagonists, may prevent overexploitation by the involved parts. We also expect environmental and ecological factors (varying in time and space), such as light-exposure, temperature variation and the presence of predators and parasitoids, have a similar role in preventing overexploitation by one of the involved partners.

Beyond describing a novel and intriguing pollination system, we aimed to characterize the balance of the plant-pollinator interaction to plants and pollinators in a single currency (i.e. in terms of fruit set) along a time series of four yrs. The data allowed us to test the assumption of the mosaic coevolution theory: that the outcome of a mutualistic interaction may vary in time. Further, with additional observations on sympatric orchid species interacting with the same group of insects, we ask whether the outcome of a plant-herbivore interaction may vary from antagonistic to mutualistic across the phylogeny and according to plants' breeding system.

Methods

Species and sites

Dichaea cogniauxiana (Aubl.) Cogn. is a small creeping epiphyte found on trunks of several tree species (one meter from the forest soil to the middle canopy) in the Atlantic forest of south-eastern Brazil[16]. The lack of pseudobulbs and the presence of single-flowered inflorescences bearing small (less than 3 cm) flowers with anchor-shaped lips are synapomorphies of the genus among the extant Zygopetalinae subtribe [17] . This orchid presents flowers with elliptic-lanceolate tepals that are primarily coloured cream-white to light rose with deep red to vine spots; the lip is punctuated in deep-red or vine violet interspaced spots (Fig. 1A). The pollinarium and its viscidium is located directly above (less than 1 mm) the stigmatic surface, and the anther cap covers the pollinarium, except for the viscidium, and bears four compressed, round pollinia that are arranged as two superposed pairs (Fig. 1B).

Beyond *D. cogniauxiana* we studied populations of *D. pendula* (Aubl.) Cogn., *Pabstia jugosa* (Lindl.) Garay, *Promenaea xanthina* Lindl., *Warrea warreana* (Lodd. ex Lindl.) C.Schweinf., *Zygopetalum crinitum* Lodd., *Z. maculatum* (Kunth) Garay and *Z. maxillare* Lodd., which occur in sympatry in the study area. *Dichaea pendula, Pab. jugosa, Pro. xanthina* and *Zygopetalum crinitum* grow as epiphytes on several host trees (*Tibouchina pulchra* – Melastomataceae, *Peltophorum dubium* and *Inga* spp. - Fabaceae) or on tree fern species (*Cyathea* spp.). *Zygopetalum maxillare* grows exclusively on the tree ferns of *Dicksonia sellowiana*, *Cyathea* spp. and *Alsophila* spp. *Zygopetalum maculatum* is terrestrial or lithophytic and thrives in sun-irradiated habitats of forest borders nearby roads or in debris among rocky outcrops in the Atlantic forest of south-eastern Brazil [16].

Field work was carried out from August 2011 to April 2015 in highland (from 800 to 1000 m a.s.l.) Atlantic forest areas (ombrophilous dense forest, Veloso et al., 1991) in Serra do Mar State Park (SMSP), south-eastern Brazil. The studied populations of *D. cogniauxiana* are from an area constituted by a mosaic of secondary (40-50 yrs old) and old (more than 50 yrs) forests between the municipalities of São Luiz do Paraitinga, Cunha and Natividade da Serra, São Paulo State (23º26'08''S-45º13'22.5''W and 23º19'55''S-45 º05'49''W, respectively). A voucher specimen of the species focus of this work and of other sympatric related species was deposited in the herbarium of the State University of Campinas (UEC; *D. cogniauxiana*: C.E.P. Nunes, 03 17-Feb-2011; *D. pendula*: E. Melo *et al.* 2555, 14-Nov-1998; R.B. Singer, 99/38 25-Nov-1999; C.E.P. Nunes, 5 19-Nov-2012; *Pab. jugosa*: C.E.P. Nunes, 13 28-Jul-2014; *Pro. xanthina*: R.B. Singer 200016 25-Jan-2000; *W. warreana*: C.E.P. Nunes 6 12-Jan-2013; *Z. crinitum*: C.E.P. Nunes 7 28-Jul-2015; *Z. maculatum*: C.E.P. Nunes 4 15-May-2015; *Z. maxillare*: R.G. Udulutsch *et al.* 674 23-Apr-2002).

Pollinators, parasitoids and the balance of the interaction

A total of 39 hours of focal observations of visitors to *D. cogniauxiana* were performed during the flowering seasons, i.e. between January and February, of 2012, 2013, 2014 and 2015 from 07:00 to 19:00 h, covering both diurnal and crepuscular periods. During each observation period, one individual bearing 1-36 flowers was observed *in situ* or recorded with a digital camera (DCR-RS68 Sony, Tokyo, Japan). Floral visitors removing and depositing pollinaria were considered to be pollinators. The behaviour of visitors and pollinators were analysed from digital videos and images of focal observations.

In order to assess the variation of the balance of the orchid-weevil-wasp interaction over space and time, fruits set under natural conditions were collected randomly from different populations at the study site, *ca.* 45 days after the flowering peak of *D. cogniauxiana*. To collect the fruits at random, we sorted individuals from each population using the function "sort" of R Statistical Computing Environment [19]. All fruits collected were dissected and checked for evidences of the presence of either weevil larva, wasp larva or weevil larva remains. To describe the dynamics of the interaction between *Montella* weevils and their parasitoid wasps, we obtained the λ value from the following equation

$$\boldsymbol{N_{t+1} = \lambda\, f(N_t, P_t)}$$

where P and N are the population sizes of the Braconidae wasp parasitoids and weevil larva inside the dissected fruits of *D. cogniauxiana*, respectively, in successive generations t and $t + 1$ (three successive generations in our study). The parameter λ is the *net* finite rate of increase of hosts in the absence of the parasitoids, which may be density-dependent. It depends on the host fecundity, sex ratio, any immigration and emigration and all the host mortalities other than parasitism itself. The function $f(N_t, P_t)$ defines the fraction N_t weevil larva escaping parasitism (modified from [20]). The resulting binary data on the presence of such evidences of interactions were also analysed using a General Linear Mixed-effects Model (GLMM) in R[19].

Breeding system and floral biology

We assessed the breeding systems of *D. cogniauxiana* and of the other sympatric species studied using controlled pollination experiments [21,22]. The pollination experiments included (i) hand cross-pollination (flowers were emasculated and hand-pollinated using one pollinarium from another orchid growing on another tree at least 10 m away), (ii) hand self-pollination (flowers were hand-pollinated using each plant's own pollinarium), (iii) spontaneous self-pollination (unmanipulated flowers were bagged without further treatment), (iv) natural pollination (flowers exposed to pollinators and florivores), and (v) weevil pollination (flowers that were observed to be pollinated by florivore weevils were bagged). The numbers of flowers and individuals in each experiment are presented in Table S1. The time, sequence and duration of anthesis (i.e., the period in which the corolla was open and male or female functional organs were present) were assessed *in situ*. The emission of flower scent throughout the day was also assessed *in situ* by smelling individual flowers or groups of flowers from a distance of 10 cm every 20 min during focal observations.

Results

Pollination and pollinator's life cycle

D. cogniauxiana was pollinated by female *Montella* sp. nova weevils, which deliberately pollinated the flowers. After self-pollinating, by manipulating pollinaria and transferring it from anthers to the stigma (Fig. 1a-b), female weevils oviposit inside the stigmatic cavity (Fig. 1c), ritualistically repeating this behaviour in each unpollinated flower they visit (video online supplementary material).Generally each fruit contained one weevil larva; however, we found one fruit containing two young weevil larvae and five fruits hosting 1 to 5 non-weevil larvae out of the 629 dissected fruits. Weevil larvae feed on developing ovules of *D. cogniauxiana* (Fig. 1d-e), causing complete loss and abortion of the fruits by the end of the larval stage (Fig. 1f; more than 20 aborted fruits were recovered with all the ovule-content consumed by weevil larva). Under natural conditions, weevil larvae infested 22.18 ± 6.07 %of the fruits (mean ± s.d., n = 4 yrs or successive reproductive seasons; with respectively103, 258, 144, 124, totalizing 629 fruits checked). Although 79.89 ± 11 % (13/18, 73/79, 18/26 and 24/28, respectively from 2012 to 2015) of the larva-containing fruits are lost, 18.19 ± 7.46 %(namely 5/18, 6/79, 8/26 and 4/28) are "rescued" by parasitoid wasps (Braconidae), which kill weevil larva before too much damage is inflicted in fruits, which, in this case, proceed to dehiscence and release the seeds. *Montella* weevils also pollinate the sympatric congener self-incompatible *D. pendula*; however, its ovules do not develop after weevil self-pollination aborting the flowers after two days (25 weevil pollinated and 25 hand self-pollinated flowers from different individuals produced no fruits).

Host-parasitoid dynamics and effects of time, population and individual on the balance of the interaction

By its turn, the outcome of the interaction of *Montella* weevils with their parasitoid wasps, in terms of fruits used by weevils and weevils killed by wasps varies over time and space (Fig. 2).Weevil-parasitoid subpopulations or patches of plants studied behave differently along time, with weevil populations always present but parasitoid populations absent in some years (Fig. 2a-c). Nevertheless, when different patches of plants are analysed together as a single population, the dynamics of weevils and parasitoids presented the two yrs of highest parasitoid densities intercalated by the two yrs of lowest host densities. The calculated *net* finite rate of increase of hosts in the absence of the parasitoids varied from $\lambda_{2012,2013}$ = 0.139 to $\lambda_{2013,2014}$ = 0.021 to$\lambda_{2014,2015}$ = 0.1 (Fig. 2d).

Neither the time (i.e. year of flowering) nor the plant individuals were related with the chance of a given fruit to be used by a pollinating-ovule-consuming weevil larva. On the other hand, the local where the subpopulation grows may influence the chance of a given fruit to host a weevil larva, with one local (Pirapitinga) having a negative effect on weevil larvae presence (GLMM, binomial, weevil infestation ~ year + individual + local, estimate $_{year}$ =- 0.29, p_{year}= 0.35, estimate $_{individual}$ = 0.008, p = 0.43, estimate $_{local}$ = - 0.44, $p_{local\ Pir}$= 0.04).

The plant individual affected the chance of a fruit to be used by a parasitoid wasp who kills weevil larvae and rescue the developing fruit (GLMM, binomial, wasp presence ~ year + individual + local + weevil presence, estimate $_{year}$ = - 2.43, p_{year}= 0.007, estimate $_{individual}$ = 0.09, p = 0.002, estimate $_{local\ PP}$= - 0.24, $p_{local\ PP}$= 0.04).

Montella *weevils and other orchid species*

At least eight sympatric orchid species observed in the study area and another species (*Catasetum fimbriatum*) observed in another site in south-eastern Brazil had their flowers visited by *Montella* weevils. Adult weevils fed on the anthers and the corolla of all the observed species while larva of different species of *Montella* also fed on ovary tissues of female flowers of *C. fimbriatum* and flowers of *Pab. jugosa*, *Pro. xanthina*, *Z. crinitum* and *Z. maculatum.* However, weevils of the genus *Montella* pollinated solely the flowers of *D. cogniauxiana*, *D. pendula* and *G. amherstiae* (Fig. S1).

Breeding system

D. cogniauxiana set fruit from spontaneous self-pollination (13.39 %) but not from emasculation. However, fruit set of natural pollination (57.47 %) was comparable to fruit set of hand selfing (47.62 %) and higher than handmade cross-pollination (37.29 %). Fruit set after hand pollination treatments and under natural pollination was significantly higher than fruit set after spontaneous self-pollination (χ^2 test, $p \leq 0.05$), characterizing a self-compatible breeding system in which the exposure to pollinators significantly increased fruit set.

Discussion

Pollination and pollinator's life cycle

Montella weevils are known florivores associated with other orchid species; however, nursery pollination behaviour was never described for this taxon. Further, this is the first report of active pollination behaviour in Coleoptera and the first report of nursery pollination in Orchidaceae, what opens a new investigation field in these two mega-diverse groups of organisms which may have part of their history of diversification intimately related to each other[23–25]. Nevertheless, pollination by beetles is well known for other Orchidaceae[26–29] and previous studies with the orchid *Grobya amherstiae* in south-eastern Brazil already suggested the active pollination behaviour in *Montella* sp. weevils [6].

This pollination system is comparable to other nursery pollination systemsdescribed[1,30,31], such as the *Silene*-moths[31,32] and *Trollius europaeus*-*Chiastocheta* flies[3,33,34], including other brood-site pollination systems involving weevils[35]. However, we describe for the first time the existence of active pollination behaviour in Coleoptera, and such a feature differentiates the system described here from all other present in literature. Therefore, this work provides new insights on evolution of active nursery pollination in pollination mutualisms involving (*Yucca*-*Tegeticula* moths and *Ficus*-Agaonid wasps, for instance)[4,36–38].

The interaction of *Montella* weevils with other orchid species involves non-mutualistic interactions, such as simple florivory with consumption of flower parts by the larvae, without pollination. In terms of fruit set, weevil-pollination proved to be advantageous compared to spontaneous selfing in *D. cogniauxiana* (Table S1). Beyond pollinating *D. cogniauxiana* and contributing to its fruit set, *Montella* weevils interact and pollinate at least two other orchid species: *D. pendula* and *Grobya amherstiae* (C.E.P. Nunes, personal observation), but the outcome of the interaction may vary from completely negative in *D. pendula* (due to its self-incompatibility) to positive in *Grobya amherstiae* (due to its self-compatibility). As the former two species are effectively pollinated by bees and *D. cogniauxiana* present floral VOCs usually found in bee-pollinated flowers (see Table S2 of Chapter 1)[39,40], we expect *D. cogniauxiana* to be also pollinated by bees.

According the distinctive features size, elytrum stripe pattern, active-pollination behaviour and the attraction by the chemical 2-methoxy-4-vinylphenol (Nunes et al., in prep.), weevils pollinating the flowers of *D. cogniauxiana*, *D. pendula* and *G. amherstiae* at SM, may be classified as *Montella* sp. nova, while non-pollinating weevils *Montella* interacting with other plant species may be grouped in other taxa, which need to be reviewed in further studies.

Host-parasitoid dynamics and effects of time, space and plant individual on the outcome of the interaction

In terms of fruit set, the outcome of the plant-weevil interaction to a given individual plant varies within different patches of a population and the chance of a weevil-infested fruit to be used and "rescued" by a parasitoid varies among different individuals. Such variations provide a material to natural selection act differentially in space and among plant individuals.

Thereby, we expect traits favouring parasitoid attraction by weevil-infested plants, such as emission of attractive volatiles[41]. Once the pressure exerted by weevils on plants fruit set varies in space, we expect the strength of the selection on individual traits would also vary among different localities[10].

Different outcomes of the interaction between other orchid species and Montella *weevils would depend on breeding system and floral traits*

Except for *D. cogniauxiana*, which produces as many fruits under natural conditions as after hand cross-pollination, all the species studied in the area exhibited pollen limitation, producing more fruits with hand cross-pollination than under natural pollination [42]. The great variation in the breeding system of the sympatric species studied reflects the diversity of reproduction strategies in the family Orchidaceae. Pollination limitation is common in orchids of the Atlantic forest [43–47]. Thereby, the fact that *D. cogniauxiana* does not present any evidence of pollination limitation may be attributed to the selfing and autonomous autogamy breeding system, as well as to the abundance, constancy and efficiency of the *Montella* sp. n. weevils as pollinators, which may guarantee that fruit set under natural pollination is higher than after hand self-pollination, for instance. Furthermore, once epiphytic orchids may have its fruit set restricted by resource limitation over time [48], more specific tests are needed to verify whether the trend observed for *D. cogniauxiana* is consistent for more than one reproductive season.

Adult *Montella* spp. weevils visited the flowers of all the populations from all the species studied at SM study site. In addition, unpollinated damaged flowers, lacking ovary tissues, were found amongst flowers of the big-flowered *Pabstia jugosa*, *Warrea warreana*, *Zygopetalum crinitum* and *Z. maculatum*. Interactions between *Montella* weevils and flowers were also observed in the fragrant bee-pollinated orchid species *Grobya amherstiae*[6], *Bifrenaria harrisoniae* and *Gomesa crispa*. Previous observations on *G. amherstiae* interaction with *Montella* weevils at another location of south-eastern Brazil[6], together with these findings suggest that *Montella* spp. interactions with flowers may result in different outcomes to plants, varying from parasitic destructive florivory to mutualistic active pollination with consumption of only part of the pollinated flowers (C.E.P. Nunes, unpublished data). Thus, in further studies in the present or in other *D. cogniauxiana* populations, we expect to observe pollinators capable of crossing, such as flies or bees, which would, thereby, prevent possible deleterious effects of inbreeding depression in this plant. Abiotic and temporal factors may influence the distribution of the interacting plant-pollinators, as predicted by the geographic mosaic theory of coevolution [8,10]. Further, the outcome of the interaction between orchid flowers and these florivory/pollinating weevils to the plants would depend on several plant traits, such as breeding system, flower morphology and flower size, as observed in other pollination systems involving ovule consumption [34,49,50].Thereby, the orchid-weevil system is an excellent model to understand the evolution of pollination mutualisms from florivory, allowing us to approach this problem from the level of individuals' natural history to the level of community ecology of plant-animal interactions.

Conclusions

We show here that host-parasitoid dynamics and inefficiency of female weevils in using all the available flowers are factors avoiding overexploitation of flowers by pollinating-herbivores. Thus, the interaction between *Dichaea cogniauxiana* and *Montella* sp. nova weevils is comparable to fig-fig wasp and the yucca-yucca moth classic examples in the sense that these interactions can be regulated by mechanisms avoiding overexploitation of the plant by the ovule-consuming pollinator [3,51].However, once no conspicuous morphological trait but only behavioural adaptations of female weevils enhance the mutualistic pollination interaction with the orchid, the orchid-weevil system may be not as ancient as the fig-fig wasp and yucca-yucca moth systems [36,37,52]. The interaction between *Montella* weevils and other orchid species involves non-mutualistic interactions, such as simple florivory with consumption of flower parts by the larva without pollination or pollination by adult females without fruit set, depending on the behaviour of weevils and on the breeding system of the plant (Fig. S1). So, the outcome of orchid-*Montella* interactions may vary across different orchid taxa according to the ability of the weevil to pollinate and to the reproductive system of the plant species. The existence of non-pollinating *Montella* species interacting with at least eight other orchid species as well as the widespread herbivore habit in Curculionidae and florivore habit in Baridinae [25,53,54] suggest that pollination behaviour in this group may have evolved from an ancestral herbivore behaviour. The different outcomes of the pollination behaviour in this orchid-weevil system studied evidence that potentially mutualistic interactions may vary widely in the outcome to their participants. Spatial-temporal dynamics, population and individual traits as well as interactions with the third trophic level may be taken in account when evaluating such outcomes.

Methods summary

Studies were conducted at the Serra do Mar State Park, São Paulo, south-eastern Brazil, from 2011 to 2015. To register the pollinators and analyse their behaviour at flowers, we performed focal observations, both directly with naked eyes and with the help of digital video cameras. In parallel we studied seven other species sympatric to *D. cogniauxiana* including the congener *D. pendula,* all of which have their flowers visited by *Montella* weevils (Fig. S1, online supplementary material).To determine the reproductive systems, we conducted controlled pollination experiments and examined fruit set under natural conditions. In order to assess the variation of the balance of the orchid-weevil-wasp interaction over a fine-scale space and time, fruits set under natural conditions were collected randomly from different patches within a population at the study sites during four fruiting seasons (2012 to 2015). All fruits collected were dissected and checked for evidences of the presence of either weevil larva, wasp larva or weevil larva remains. To test the effects of variation in time (year) and patch, the resulting binary data on the presence of such evidences of interactions were them analysed using General Linear Mixed-effects Models (GLMMs) in R[19].

Supplementary Information is linked to the online version of the paper.

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Figures

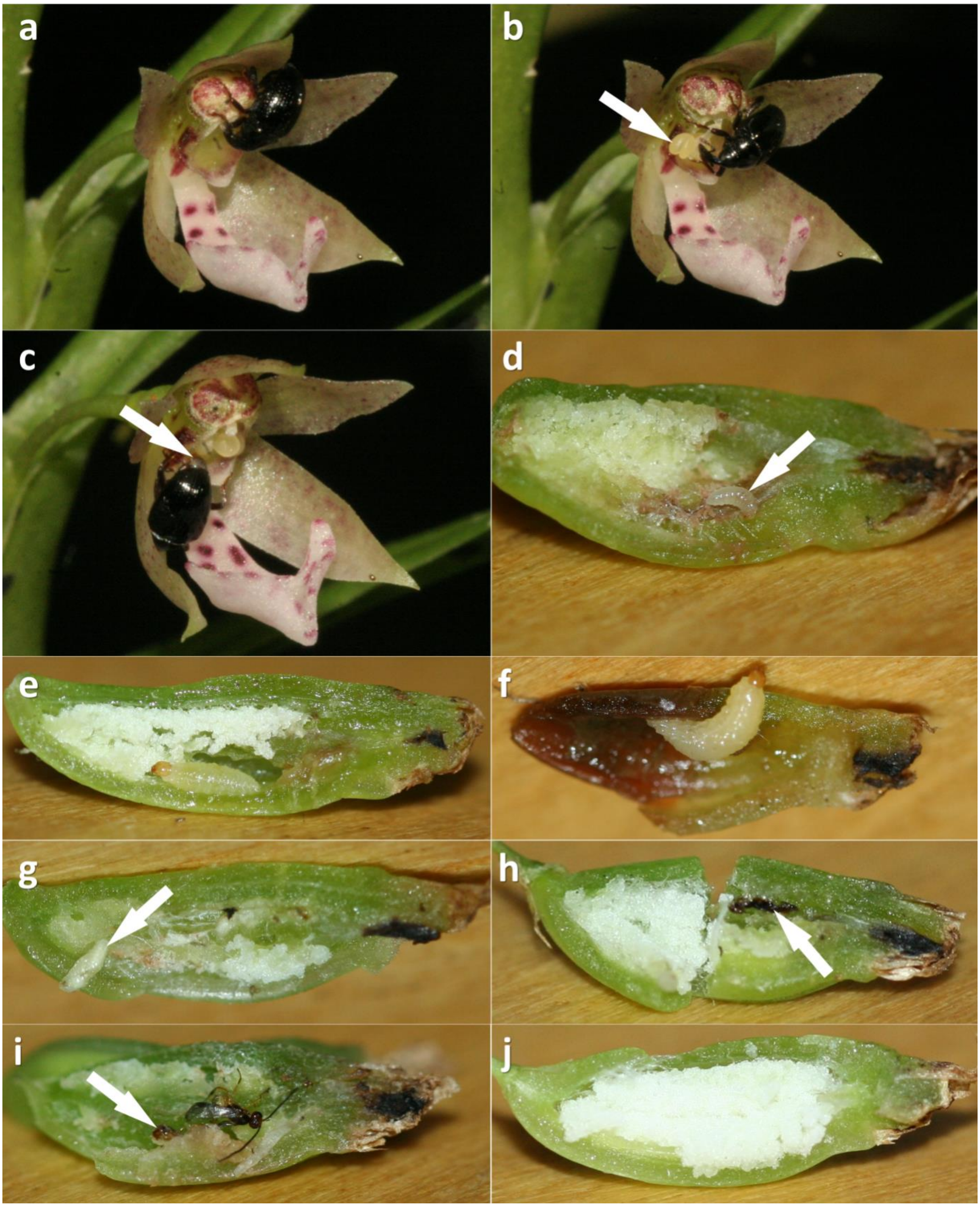

Figure 1 | Nursery pollination and life cycle of *Montella* sp. nova weevil and its parasitoid in flowers and fruits of the orchid *Dichaea cogniauxiana*. a, *Montella* sp. nova female weevil initiating active pollination by inserting its rostrum under the anther cap. **b,** *Montella* sp. nova female weevil manipulating the pollinarium (arrow) while pushing it down towards the stigmatic cavity. **c,** *Montella* sp. nova female weevil inserting its ovipositor (arrow) to lay an egg in the stigmatic cavity after pollination. **d,** Young larva of *Montella* sp. nova (arrow) inside a fruit of *D. cogniauxiana* with part of the fruit content consumed. **e,** Older larva of *Montella* sp. nova. **f,** Larva of *Montella* sp. nova at the last stage of larval development in an emptied fruit aborted by the plant. **g,** Pupae of the parasitoid Braconidae sp. wasp (arrow) in a fruit with part of its content consumed and remains of a *Montella* sp. nova larva. **h,** Remains of a *Montella* sp. nova larva (arrow) in a fruit with evidences of parasitoid attack. Note the remaining content of the fruit. **i,** New-born adult Braconidae sp. parasitoid in a fruit with remains of a consumed *Montella* sp. nova larva. **j,** Fruit without evidences of infection by weevils one month after pollination.

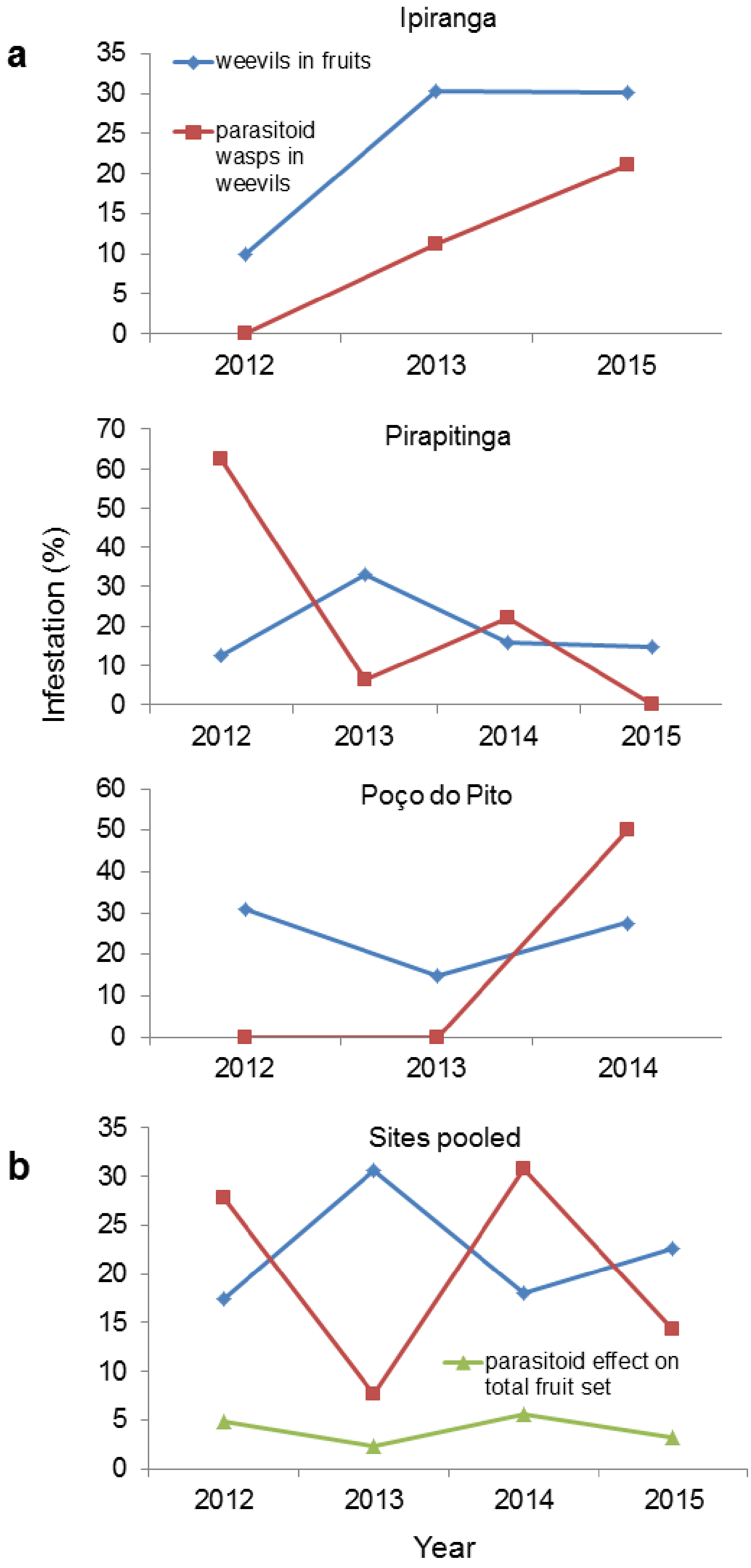
a
Ipiranga
weevils in fruits
parasitoid wasps in weevils
0
5
10
15
20
25
30
35
2012
2013
2015
Pirapitinga
0
10
20
30
40
50
60
70
2012
2013
2014
2015
Infestation (%)
Poço do Pito
0
10
20
30
40
50
60
2012
2013
2014
b
Sites pooled
parasitoid effect on total fruit set
0
5
10
15
20
25
30
35
2012
2013
2014
2015
Year

Figure 2 | Temporal dynamics of the infestation of fruits of the orchid *Dichaea cogniauxiana* by weevils (*Montella* sp. nova) and of weevil larva by their parasitoid wasps (Braconidae sp.).a, Population dynamics of *Montella* weevil and their parasitoid in different patches in a single population from 2012 to 2015. Data lacking for 2013 in Ipiranga and 2015 in Poço do Pito sites. **b,** Population dynamics of pooled samples and the impact of parasitoids on plants fruit set in percentage of fruits “rescued” from weevil larvae from 2012 to 2015.

Supplementary material

Can orchids be deceived by weevil pollinators? A novel nursery pollination system provides insights on the evolution of pollination from herbivory

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Tables

Table S1 | Controlled pollination experiments results of *D. cogniauxiana* and eight sympatric orchid species from south-eastern Brazil. Percentage of fruit set (number of fruits / number of flowers) and number of flowers (in parenthesis) treated in controlled pollination experiments at different locations in south-eastern Brazil. Data for all species were collected at Serra do Mar, with additional data for *Z. crinitum* at Serra do Japi (SJ) and for *Z. maxillare* at Itatiaia National Park (INP).

	D. cogniauxiana	*D. pendula*	*Pab. jugosa*	*Pro. xanthina*	*W. warreana*	*Z. crinitum*	*Z. crinitum* SJ	*Z. maculatum*	*Z. maxillare*	*Z. maxillare* INP
emasculation	0 (38) a	-	-	0 (4)	-	0 (3)	0 (32)	0 (5)	5.88 (17)**	0 (8)
spontaneous self-pollination	13.39 (112) b	0 (32)	-	-	0 (18)	0 (12)	0 (32)	0 (9)	100 (6)	0 (8)
hand self-pollination	47.62 (42) d	0 (24)	40 (5)	66.66 (9)	83.87 (31)	9.52 (21)*	0 (32)	79.31 (58)	15.38 (13)	22.22 (18)
hand cross-pollination	37.29 (59) c	45.45 (33)	50 (4)	0 (9)	92 (25)	88.89 (9)	46.87 (32)	78.33 (60)	46.15 (13)	100 (9)
natural pollination	57.47 (395) d	5.6 (125)	0 (6)	0(12)	15.38 (26)	6.8 (103)	3.04 (197)	4.88 (205)	17.71 (96)	16.66 (549)

Different letters in each column represent significant differences in the Chi-square ($p \leq 0.05$) test performed when applicable.

* Only inflorescences without cross-pollinated flowers set fruits after self-pollination.

** Only inflorescences with hand pollinated flowers set fruits after emasculations.

Table S2 | Habit, flower size and lifespan, floral resources and animal pollinators of other eight orchid species sympatric to *D. cogniauxiana* at Serra do Mar, south-eastern Brazil.

	D. cogniauxiana	*D. pendula*	*Pab. jugosa*	*Pro. xanthina*	*W. warreana*	*Z. crinitum*	*Z. maculatum*	*Z. maxillare*
Habit	epiphyte	epiphyte	epiphyte	epiphyte	terrestrial	epiphyte	terrestrial	epiphyte
Floral resource	developing ovules	perfume	absent	absent	absent	absent	absent	absent
Flower diameter (cm)	< 1	< 2	> 4	> 3	> 3	> 4	> 3	> 3
Flower lifespan (days)	2-4	2	> 10	> 6	> 6	> 14	> 14	> 14
Pollinators	*Montella* sp. n.	Male euglossine and *Montella* sp. n.	Unknown	Unknown	*Bombus brasiliensis*	*Xylocopa* sp.	*Bombus* spp.	*Bombus* spp. and *Centris confusa*

Table S3 | Number of individuals and flowers (in parenthesis) used in the controlled pollination experiments and hours of focal observations in *D. cogniauxiana* and eight sympatric orchid species from the Atlantic forest of south-eastern Brazil. We present numbers for all the species studied at Serra do Mar, and discriminate the numbers for *Z. crinitum* and *Z. maxillare* additionally studied at Serra do Japi (SJ) and Itatiaia National Park (INP), respectively.

	D. cogniauxiana	*D. pendula*	*Pab. jugosa*	*Pro. xanthina*	*W. warreana*	*Z. crinitum*	*Z. crinitum* SJ	*Z. maculatum*	*Z. maxillare*	*Z. maxillare* INP
emasculation	5(38)	2(2)	-	2(4)	-	2(3)	16(32)	1(5)	4(17)**	13(8)
spontaneous self-pollination	11(112)	7(32)	-	-	3(18)	4(12)	16(32)	2(9)	1(6)	13(8)
hand self-pollination	7(42)	16(24)	2(5)	4(9)	9(31)	10(21)*	16(32)	19(58)	3(13)	13(18)
hand cross-pollination	10(59)	18(33)	4(4)	4(9)	9(25)	7(9)	16(32)	18(60)	5(13)	13(9)
natural pollination	34(395)	52(125)	3(6)	6(12)	7(26)	13(103)	15(197)	30(205)	14(96)	57(549)
Focal observations (h)	39	84	33	78	19	67	83	71	89	41

Figures

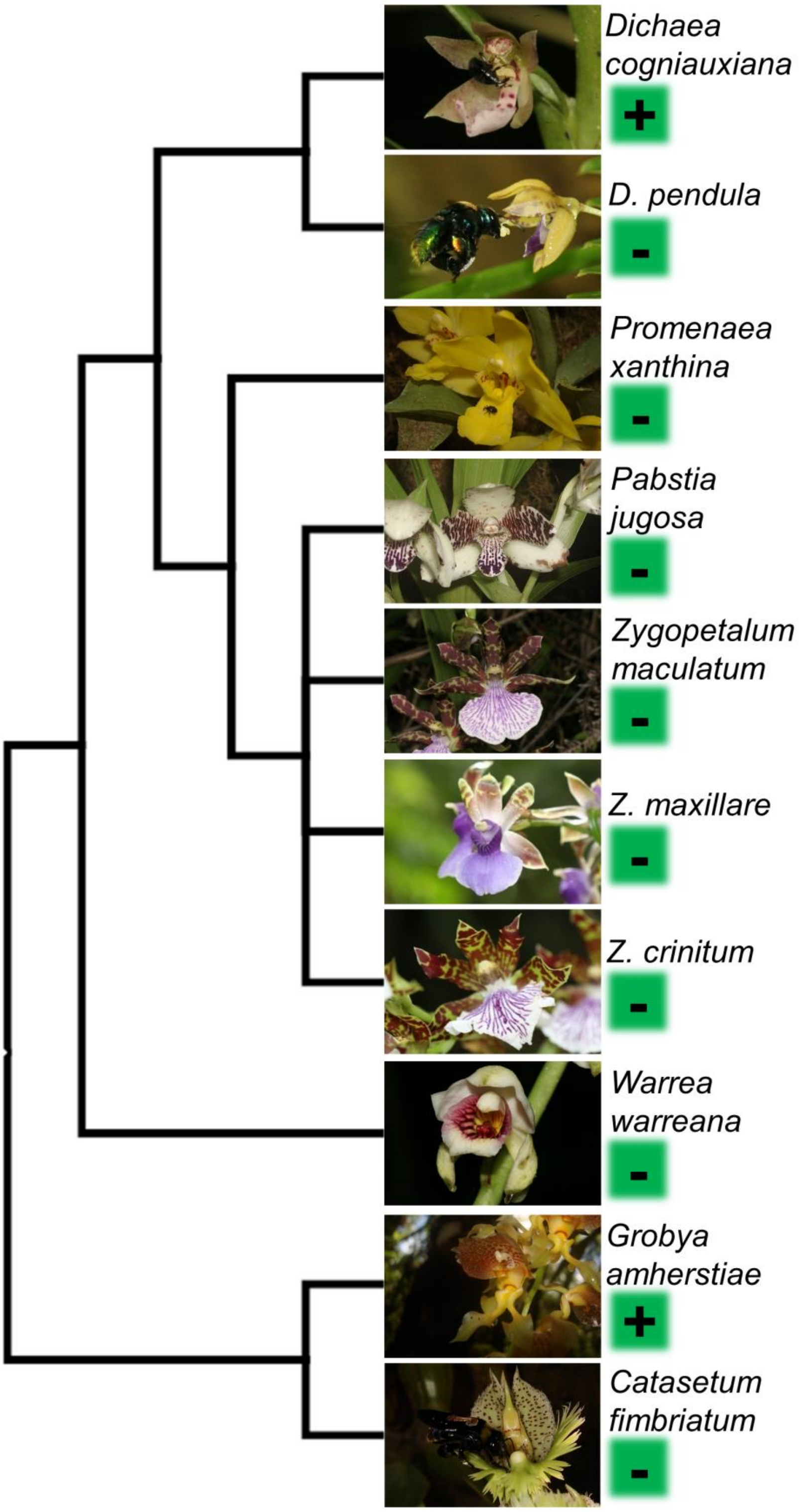
Dichaea cogniauxiana
+
D. pendula
-
Promenaea xanthina
-
Pabstia jugosa
-
Zygopetalum maculatum
-
Z. maxillare
-
Z. crinitum
-
Warrea warreana
-
Grobya amherstiae
+
Catasetum fimbriatum
-

Figure S1 | Phylogenetic relationships and the outcome of the interaction between orchid species and *Montella* weevils. The interactions were observed and the outcome on the fruit set was inferred according the species breeding system and the presence of pollination behaviour in the flower-visiting *Montella* weevils. All the studied species were observed to be pollinated by bees, except for *Dichaea cogniauxiana*, which was only pollinated by *Montella* weevils, *Pabstia jugosa* and *Promenaea xanthina*, whose pollinators could not be recorded.

“The Orchids have been splendid sport, though at present I am fearfully sick of them. “

(Darwin, 1862)

Chapter IV

Orchids' imitation game: floral scents of *Catasetum cernuum* and *Gongora bufonia* suggest convergent evolution to a unique pollination niche

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Orchids' imitation game: floral scents of *Catasetum cernuum* and *Gongora bufonia* suggest convergent evolution to a unique pollination niche

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ABSTRACT

Floral volatile organic compounds (VOCs) are important signals in plant-pollinator communication. Thus, floral VOCs are subjected to pollinator-mediated selection that may favour individual plants attracting the most effective pollinators. Occasionally, phylogenetically unrelated plant species would converge to similar floral scent signalling patterns to attract a given pollinator representing a common adaptive pollination niche. Our field observations in Atlantic forest areas of south-eastern Brazil and reports from literature provided the information that *Catasetum cernuum* and *Gongora bufonia* are exclusively pollinated by males of a single orchid-bee species, *Eufriesea violacea.* In order to test the hypothesis that these two orchid species converged to similar scents attracting males of a single euglossine as pollinator, the floral scents of the two species were characterized using headspace techniques, then the similarity of scents were tested using multivariate analyses. Floral scents of both species do not differ significantly from each other, being a blend of VOCs usually found in other euglossine-pollinated species. These species share some unusual floral VOCs, such as *(E)*-epoxyocimene, a rare constituent of floral scents, found in few other orchid species, all of them occurring outside of the distribution range of *C. cernuum*, *G. bufonia* and *E. violacea*. Thereby, the authors hypothesize that *(E)*-epoxyocimene or a mixture of it with other floral VOCs determines the specific association between these two orchids and their exclusive euglossine pollinator.

KEYWORDS

chemical ecology; convergent evolution; *Eufriesea*; Euglossini; Cymbidieae; Orchidaceae.

1.0 INTRODUCTION

Communication between plants and pollinators usually involves floral volatile organic compounds (VOCs) and the study of these chemicals has been important to understand ecology and evolution of plant-pollinator interactions (Raguso, 2008; Raguso et al., 2015; Schiestl, 2015). Together with visual cues, VOCs are important in both long-distance attraction to and short-distance recognition of floral resources by pollinators (Chittka and Raine, 2006; Leonard and Masek, 2014; Wright and Schiestl, 2009).

Floral scents are constituted by more or less complex mixtures of VOCs specific to each plant species, in which each compound may vary in its presence/absence and relative proportion (Knudsen et al., 1993, 2006). Floral scent signalling can enhance sexual reproduction in plants by mediating plant-pollinator interactions and thus undergoes natural selection that may favour individual plants attracting the most effective pollinators (Schiestl and Johnson, 2013; Schiestl, 2015). Consequently, floral scents are determinants in the specialization of many plant species in a given pollinator guild or even in a single species of pollinator (Ackerman, 1983; Schäffler et al., 2015). Occasionally, unrelated plant species would converge to similar floral scent or other signalling patterns to attract a given pollinator representing an adaptive pollination niche (Gang, 2005; Johnson and Raguso, 2015; Johnson, 2010).

Convergent evolution in colour and shape of flowers have been demonstrated to occur between several Oncidiinae orchids and sympatric oil-offering Malpighiaceae (Papadopulos et al., 2013). Additionally, flowers from several distinct angiosperm families and a stinkhorn fungus have converged to the same odour signals in order to attract carrion flies which namely pollinate and disperse this distant-related organisms (Johnson and Jürgens, 2010). In opposition, pollinator-mediated selection towards distinct pollination niches does not proved as important as phylogenetic constraints in explaining the diversity of floral scents of South

African oil-secreting orchids of the tribe Diseae (Steiner et al., 2011). Additionally other cases of convergent evolution and repeated evolution in floral scents are reviewed by Gang (2005).

The family Orchidaceae comprises huge morphofunctional and chemical floral diversity which can be linked to events of adaptive radiation towards diverse habitats and distinct pollinator groups(Givnish et al., 2015; Gravendeel et al., 2004; Kaiser, 1993; Mondragón-Palomino and Theissen, 2009). Thus, orchids may be excellent to study the relationships among plants and their pollinators mediated by floral volatiles (Bronstein et al., 2014). Consequently, ecological studies *in situ* are essential to understand the actors and roles that are involved in plant-animal interactions as mediated by volatile compounds.

The orchids *Catasetum cernuum* (Lindl.) Rchb.f. and *Gongora bufonia* Lindl. are native from the Atlantic forests of south-eastern Brazil. Although their geographic range may not be the same, they share their habitats in the forests all along tropical eastern Brazil, mainly in the Serra do Mar Mountain Range of the States Espírito Santo, Minas Gerais, Rio de Janeiro, Santa Catarina and São Paulo(Barros et al., 2014; INCT, 2015). Additionally, they are both epiphytic herbs growing from the middle to the high canopy of tropical rainforest and riparian forests (Hoehne, 1933). Thereby, they may be subjected to converging selective pressures from their habitat, including selective pressures from a given assemblage of pollinators.

The relation between *C. cernuum* and *G. bufonia* and their pollinator (i.e. male *Eufriesea violacea*) has been reported in the literature since Hoehne (1933). However, since the identity of pollinators are based on isolated reports, this work aims to study the pollinator specificity and characterize the floral scent of the euglossine-pollinated *C. cernuum* and *G. bufonia* in order to test the hypothesis that these two orchid species converged to similar scents attracting a unique euglossine-pollinator.

2.0 METHODS

2.1 Study species and sites

Both species studied are epiphytic herbs native to the south-eastern Brazilian Atlantic forest (Fig. 1) (Barros et al., 2014). As all the species in the genus, *C. cernuum* is a monoecious plant which produces female and male flowers (Fig. 1A-C) separately in time in a given individual with rare cases of hermaphroditic flowers or inflorescences (Milet-Pinheiro et al., 2015; van der Pijl and Dodson, 1969). On the other hand, *G. bufonia* present hermaphroditic flowers (Fig.1D-F) and may produce fruits from both self- and cross-pollinations(Adachi et al., 2015). However, both species depend on euglossine pollinators to set fruit (Adachi et al., 2015; Martini et al., 2003).

The two species present complex distinct pollination mechanisms enhanced by idiosyncratic morphological adaptations typical to their respective genera (Adachi et al., 2015; Nicholson et al., 2008). The mechanism of pollination is distinct for both species. In *C. cernuum*, as in all other species in the genus, pollinaria attachment to pollinators occurs through a catapult mechanism triggered when, while collecting perfumes from the lip, the euglossine-bee touches the antennae of male flowers (Fig. 1A). Afterwards, the pollinarium is explosively launched towards the body of the bee where it normally sticks on the scutum (Nicholson et al., 2008). On the other hand, in *G. bufonia*, pollinaria attachment occurs through a “slip and fall” mechanism (Fig. 2B-D)(Adachi et al., 2015; Martini et al., 2003).

According data from the literature and unpublished pollinaria-carrying data from R.L. Dressler, both species rely solely on males of the orchid-bee *Eufriesea violacea* for pollination (Adachi et al., 2015; Davies and Stpiczyńska, 2009; Hoehne, 1942, 1933; van der Pijl and Dodson, 1969).

Field observations were carried out at two different locations of south-eastern Brazil in which the species studied occur in sympatry: the natural reserve of Serra do Japi (SJ) and

Serra do Mar State Park (SM). The SJ study site is located within the boundaries of the municipality of Jundiaí (23º11'S, 46º52'W; 700 – 1300 m a.s.l.), while SM (23º26'08"S - 45º13'22.5"W and 23º19'55"S - 45 º05'49"W, from 800 m to 1000 m a.s.l.) is located in the Serra do Mar mountain range between the municipalities of São Luiz do Paraitinga, Cunha and Natividade da Serra, São Paulo State.The SJ is characterized by a mesophytic semi-deciduous forest, while the SM is characterized by secondary (40-50 yrs old) montane ombrophilous dense forest (IBGE, 2012). Observations at the SJ were carried out from 1998 to 2000 and from 2010-2011, while field work at SM was carried out from 2012 to 2015 during part of the rainy season (from October to March).Voucher specimens of the plants (*C. cernuum:* E.R. Pansarin 548, 01-Nov-1999; Unknown collector UEC 140082, Oct-1998; R.B. Singer UEC 140279, 30-Oct-1997. *G. bufonia:* J.A. Lombardi 1, 06-Dec-1990; F. de Barros et al. 29832, 10-Nov-1993; R.B. Singer 99/31 and 99/39, 25-Nov-1999) are deposited in the herbarium of the State University of Campinas (UEC).

2.2 Volatile collection and characterization of floral scent (headspace analysis)

For the *C. cernuum* occurring at the SJ, collection and characterization of floral VOCs were performed as follows.For the Headspace solid-phase micro extraction (HS-SPME) of volatiles compounds of*C. cernuum*from SJ, the flowers were placed separately in a 20 mL glass vial closed and submitted to a constant temperature (35 °C) for 60 min. In this period, the fused silica fiber coated with 100µm polydimethylsiloxane (PDMS) (Supelco Inc., Bellefont, PA) was introduced into the vial and exposed to the floral headspace. The analyses of volatile compounds were performed using a gas chromatograph coupled to a mass spectrometer (GC-MS) QP2010 Shimadzu. An electron ionization mass spectrometry (EI-MS) detector has been operated under ion source temperature of 250 °C, a trap emission current of 60 µA and a 70eV ionization energy. The global run time was recorded in full scan mode (40-500 *m/z* mass range) and a scanning ratio of 0.30 scan s^{-1}. Compounds were thermally desorbed from the SPME-fiber in the injector port for 2.0 min at 250 °C, with the port in splitless injection mode. The compounds separation was performed on a DB-5MS capillary column (J&W Agilent) of 30 m X 0.25 mm, film thickness 0.25 µm, with He (79.7 kPa) as a carrier gas at a flow rate of 1.3 ml min^{-1}. The GC oven temperature was initially 60 °C, and then linearly rose by 3 °C min^{-1} to 240 °C during 60 min. The chromatographic data were analyzed by GC-MS Solution software (Shimadzu) and volatile compounds were identified by screening the NIST 62, WILEY 7 and FFNSC 1.3 libraries for comparison of MS spectra. In addition, Kovats retention indexes (KI) of each compound were calculated using data of the injection of a homologous set of *n*-alkane (C_9-C_{22}) according Kovats formula (Robards et al., 1994). Mass spectra similarities combined with KI were used for compounds identification. Peak areas from total ion current (TIC) chromatograms were integrated to determine the relative amounts of each compound.

For one of the *C. cernuum* samples and the five *G. bufonia* samples, collection and characterization of floral VOCs were performed as follows. Individuals of the two orchid species kept in the greenhouses of the Botanical Garden of Munich were olfactorily evaluated and verbally described. To trap a scent sample, the inflorescence of the respective species was placed in a glass vessel of adapted size and shape. The scented air surrounding the flower was drawn with the aid of a small battery operated pump (personal air sampler SKC 222-4) for a period of 2 to 4 hours, depending on intensity of the scent, through an adsorption trap containing 3 mg of Porapak Super Q. Subsequently, the adsorbed scent was recovered by elution with 20 to 60 µL high grade hexane/acetone 5:1 mixture. The eluate was analyzed directly by injecting 1.5 µL into the GC (Carlo ErbaFractovap 4160) or GC-MS (ThermoFinnigan Voyager Mass Spectrometer combined with a Trace GC and the Xcalibur software). The analyses were made on a DBWAX column (J&W Scientific) 30 m x 0.32 mm i.d., film thickness 0.25 µm. Compounds were identified by comparison of their mass spectra and retention times with authentic reference samples available from the collection of reference compounds or specially synthesized in connection with this investigation (see also Kaiser and Tollsten, 1995).

All the volatile samples were collected on sunny days during the periods when scent were perceptible to the human nose both in field and in greenhouse conditions (from 10:00 to 13:30h).

2.3 Floral scent statistical analysis

Because the 'individuals × VOCs' matrix of floral scents met the assumption of multivariate normality of variances (Shapiro–Wilk normality test, W = 0.2, $p = 1.23\ e^{-10}$, test performed with the MVNORMTEST package [Jarek 2012] in R), but not the assumption of multivariate homogeneity of group dispersions (variances, ANOVA $F_{1,5} = 0.44$, $p = 0.66$, test performed with the VEGAN package [version 2.0–5; Oksanen et al. 2012] in R), a non-parametric tests was used to characterise and test for differences between the sample of *C. cernuum* and the five samples of *G. bufonia*. Hellinger transformation, a relativisation by row (sample unit) totals followed by taking the square root of each element in the matrix, to make the floral scent data containing many zeros (e.g., compounds completely absent in certain species, but present in others) suitable for multivariate analysis (Legendre and Gallagher, 2001; Legendre and Legendre, 1998). A non-parametric multiple response permutation procedure (MRPP) with the average Bray–Curtis distances among samples weighted to group size and 999 random permutations (McCune et al., 2002; Mielke and Berry, 2001) was first conducted to test the null hypothesis of no difference in floral scent (relative amounts, in %) among species. The MRPP test was performed with the VEGAN package (version 2.0–5; Oksanen et al. 2012) in R. An analysis of similarities (ANOSIM) using the average Bray–Curtis distances among samples and 1000 permutations with the VEGAN package (version 2.0–5; Oksanen et al. 2012) in R was also conducted as an alternative way to statistically test whether there is a significant difference in floral scent composition among Zygopetalinae species. To detect floral scent compounds whose presence is statistically associated with certain species, an indicator compound analysis (ICA) with 999 random permutations was performed. The computed indicator value of each compound reflects both its relative abundance (specificity – 'A', the probability that the odour compound belongs to the target species) and its relative frequency (fidelity – 'B', the probability of finding the odour compound in other species). The associated *p*-values determine whether specific compounds are significant indicators of certain species (De Caceres and Legendre, 2009; Dufrêne and Legendre, 1997). The ICA was performed with the INDICSPECIES package (De Caceres and Legendre, 2009) in R.

2.4 Chemical baits

In order to infer the plant-pollinator interactions from pollinaria-carrying by male orchid-bees and confirm the exclusivity of *E. violacea* as the pollinator of the studied species, censuses of these insects were performed using chemical baits at SJ. Commercially obtained VOCs (eucalyptol, linalool, indole, benzyl alcohol, benzyl benzoate and vanillin) were used at SJ in order to attract any male euglossine bees carrying pollinaria of any orchid species. Each substance was applied independently, onto separate 6 x 6 cm pads of herbarium blotter paper and pinned on tree trunks. The quantity and frequency of substance reposition to maintain attractiveness varied from 30 min to two hours and depended on the evaporation rates of each VOC.

The censuses were carried out from October to December, the flowering period of *C. cernuum,* between 1998 and 2000, totalling about 90 h. The daily period of census was from 8:00 to 16:00 h. The censuses were undertaken in alternate days along the flowering period of

C. cernuum and mainly in sunny days, because cloud cover and precipitation seem to decrease the activity of euglossine bees on chemical baits (Armbruster and Berg, 1994; Inouye, 1975; Williams and Whitten, 1983).

In the SJ the insects collected either when visiting the flowers or with chemical baits were identified and vouchers were deposited at the "Pollinator Collection" of the LBMBP laboratory of the Departamento de Biologia, Faculdade de Filosofia, Ciências e Letras de Ribeirão Preto, University of São Paulo, Brazil.

2.5 Focal observations of pollinators

At the SJ focal observations were performed on male inflorescences of *C. cernuum* from 19-23 October 1998 and 27-30 October 2000. In both flowering seasons the daily period of observation was from 08:00 to 17:00 h, totalizing 81 hours. At the SM, focal observations of pollinators and their behaviour were performed in October and December 2011 and October 2013 in *C. cernuum* and in December 2014 in *G. bufonia*, during the rainy season but on sunny days from 10:00 to 16:00h. In *C. cernuum*, two hours of observations distributed in two days were performed also and only in male inflorescences, due to the rarity of the female ones. In *G. bufonia*, 10 hours of observations were distributed in three days. During the observations, the behaviour of the pollinator was recorded with the help of digital cameras (Canon, EOS20D and Sony DCR).

3.0 RESULTS

3.1 Floral scent

To the human sense of smell, *C. cernuum* presented a strong floral scent only during the late morning (10:00 to 13:30h) and no perceptible floral scent or only an acetic acid smell during the rest of the day. On the other hand, *G. bufonia* presented a weak floral scent in the warmer hours of the morning and early afternoon (10:00 to 13:30h). The scent of both species was only perceptible during relatively hot and sunny days, but it was still very weak for human perception.

A total of 26 VOCs were identified in the samples of the floral headspace of *C. cernuum* and *G. bufonia*: monoterpenes dominated the scent (21 VOCs; 81.64 ± 29.8 %), accompanied by relatively small amounts of non-terpenoids or shiquimate derivatives (4 VOCs; 0.4 ± 0.56 %) and acetic acid (Table 1). Both species shared a blend of 11 identical VOCs and, for instance, the 84% of the components of the floral scent of *C. cernuum* were substances also present in the floral scent of *G. bufonia.* The multiple response permutation procedure (MRPP) indicated that floral scents (relative amounts, in %) were not significantly differentiated among these species (MRPP, $A = 0.21$, $d_{obs} = 24.05$, $d_{exp} = 30.49$, $p = 0.09$). Similarly, the ANOSIM showed no significant difference among samples of both species (ANOSIM, $R = 0.8$, $p = 0.17$).Additionally , the analysis of levels of variance dispersion with Euclidean distances among all floral scent samples showed that there were no significant differences within the samples of both species studied (ANOVA $F_{6, 42}= 0.99$, $p = 0.45$).

The sample of *C. cernuum* presented a set of exclusive VOCs as well as the samples of *G. bufonia*. The monoterpenoids *p*-cymene, α-thujene, sabinene, 1,2-epoxymyrcene, 3,10-epoxymyrcene, and the acetic acid were exclusive components of the scent of *C. cernuum.* Conversely, the non-terpenoids VOCs 4-methylanisole, phenylethyl alcohol and benzyl benzoate and the monoterpenoids 6-methyl-3,5-heptadien-2-one, rosefuran, epoxy rosefuran, 3,7-dimethyl-octa-2, *(E)*-4,*(E)*-6-trienal, 4-methyl-ethyl-pent-2-enolid were present exclusively in *G. bufonia* floral scent (Table 1). The exclusive VOCs from both species were present only in small fractions (< 5 % of each VOC in each sample) and composed no more than 8.1 % of the floral scent of any sample. However, according to the ICA, no VOC could be significantly associated exclusively to any of the studied species.

3.3 Pollination

At SM study site, males of the orchid-bee *Eufriesea violacea* were the only insects observed removing and deposing pollinaria of *G. bufonia* while collecting perfumes from the lip and no other bee species were even observed visiting flower of this species. In *G. bufonia*, pollination occurs through a "slip and fall" mechanism in which, while collecting perfumes from the lip, the male *E. violacea* slips and falls with scutellum on the stigmatic/viscidium region of the column, deposing and removing pollinaria during its fall. The two lip lateral appendages function as handrails, directing the insect as it slips its middle legs down on it during its tumble(Fig. 2B-D; click the linkhttps://www.youtube.com/watch?v=BDW23XUGV3s%20to watch an pollination event recorded *in situ*).

During observations at SM, no floral visitors were recorded in the flowers of *C. cernuum.* Yet at SM, male *E. violacea* were observed visiting flowers removing and carrying pollinaria of another orchid, *Dichaea pendula* (Aubl.) Cogn. (Fig. 3F).During observations at SJ study site, male *E. violacea* were also the only floral visitors recorded in the flowers of *C. cernuum*.

With the use of chemical baits at the SJ, several species of euglossine bees carrying pollinaria of orchid species were collected. However, males of *Eufriesea violacea* were attracted exclusively by eucalyptol and vanillin, some of the individuals collected at the chemical baits were carrying pollinaria of the following three orchid species: *C. cernuum*(Fig. 3A-B), *G. bufonia*(Fig. 3C-D) and *Bifrenaria harrisoniae* (Hook.) Rchb.f. (Fig. 3E). Additionally, one of those male *E. violacea* carried two pollinaria of *G. bufonia* as well as one viscidium plus caudicle of *C. cernuum*(Fig. 3D), and another carried two pollinaria of *B. harrisoniae* together with one of *G. bufonia* (Fig. 3E).

C. cernuum and *G. bufonia* deposit their pollinaria in different regions of the bee body. *Catasetum cernuum* get its pollinaria attached in the scutum while *G. bufonia* attaches its pollinaria in the scutellum (Fig. 3A-D). However, due to its larger size and distinct position, the pollinia of *C. cernuum* may partially superpose the one of *G. bufonia*. By its turn, *G. bufonia* lays its pollinaria in same region of the bee body that *B. harrisoniae* uses to lay its own pollinaria, the scutellum (Fig. 3E). Yet, *D. pendula* uses the head of the bee to attach its pollinarium (Fig. 3F).

4.0 DISCUSSION

4.1 Floral scent

Among the 11 VOCs shared by the *C. cernuum* and *G. bufonia, (E)*-o and *(E)*-epoxyocimene are major components (with more than 10% in relative amount) in most of the samples of both species, being present in all analysed samples in relative amounts varying from 2.8 to 76.7 %(Table 1). The presence of these both VOCs in these species floral scents is noteworthy.

The VOC *(E)*-ocimene dominates the floral scent of *C. cernuum* and *G. bufonia* (with relative amounts of 46.2 ± 24.32 and 25.73 ± 15.86 % respectively). This VOC has proven to enhance antennal electrophysiological responses in electroantennogram (EAG) experiments with four other orchid-bee (*Euglossa*) species native from Central America (Eltz et al., 2006)and, thus, may have a hole in the attraction of these bees (El-Sayed, 2012).

On the other hand,*(E)*-epoxyocimene is an unusual VOC in orchid flowers, being found only in 7 out of 243 species in Stanhopeinae. All the genera within this subtribe were investigated covering at least 78% (191 out of 243) of the entire group (G. Gerlach, unpublished data). The other species which presented*(E)*-epoxyocimene in the floral scent are the Stanhopeinae*Coryanthessenghasiana*G.Gerlach,*Gongoraarmeniaca*(Lindl.) Rchb.f., *G. ionodesme* G.Gerlach, *G. horichiana* Fowlie*, Stanhopea florida* Rchb.f. and *S. radiosa* Lem.. In those species, the relative amounts of this VOC varies from 1 to 5% (G. Gerlach, unpublished data). However, those other Stanhopeinae species emitting*(E)*-epoxyocimene are not recorded in the geographic range of their close relative *G. bufonia* neither in the area of occurrence of *E. violacea* (Barros et al., 2014; Moure et al., 2012). Additionally, the presence *(E)*-epoxyocimene distinguishes the scents of *C. cernuum* from the scents of all its studied congeners and most of the orchid species studied (G. Gerlach, unpublished data; Knudsen et al., 2006; Milet-Pinheiro et al., 2015).

Except for the acetic acid present in *C. cernuum* flowers, the exclusive components of the floral scents of each species belong to the same biochemical groups (non-terpenoids – or shiquimate derivatives –and monoterpenes), being similar in their molecular structure (Table 1) and likely having similar behavioural functions in the cognitive system of euglossine pollinators(Eltz et al., 2006; Milet-Pinheiro et al., 2015).

Therefore, we predict that orchid species that emit this floral volatile occurring outside the range of *E. violacea* or flowering when this orchid-bee species is not active in its habitat would be pollinated by other orchid-bees, for instance, other species of *Eufriesea, Euglossa* or *Eulaema.*

4.2 Pollination and evolution

Males of *E. violacea* also visit and pollinate flowers of other orchid species in the area of occurrence of *G. bufonia* and *C. cernuum*, such as *Cirrhaea longiracemosa* Hoehne, *Dichaea pendula* and *Bifrenaria* sp. Lindl. at SM (C.E.P. Nunes, L.M. Pansarin and R.L Dressler unpublished data), and *Bifrenaria harrisoniae* and *Cirrhaea dependens* (Lodd.) Loudon at SJ (Pansarin et al., 2006). Thus least five sympatric orchid species (namely *B. harrisoniae*, *C. cernuum*, *Cirr. longiracemosa*, *D. pendula* and *G. bufonia*) use male *E. violacea* as pollinator. Since all these *E. violacea*-pollinated species flower during the period from November to January of the rainy season, mechanism to avoid pollination interference should be present in this guild. As previously observed in other systems by Roubik and Ackerman(1987), some of the '*E. violacea*-orchids' use distinct parts of the pollinators body to depose their pollinaria, what would avoid any negative effect of reproductive interference or competition for pollinators (Mitchell et al., 2009; Muchhala and Thomson, 2012; Tokuda et al., 2015). However, in the specific case of *B. harrisoniae* and *G. bufonia*, both which use the same body part of the bee to lay their pollinaria, the avoidance of reproductive interference would be achieved by other means, such as different sizes and shapes of pollinia which fits the stigmatic cavity in a key-lock mechanism, for instance. However, more studies on reproductive interference and pollination mechanisms in orchids are needed to test such assumption.

Thereby, in the case of the species studied, there is an asymmetrical dependence of orchids on orchid-bees, in which an orchid species may depend on one of few orchid-bee species for pollination but the orchid-bees may not depend on the pollinated orchids for their survival (Pemberton and Wheeler, 2006; Ramírez et al., 2011; Singer and Sazima, 2004).

Beyond the observations of this work, other researchers collecting at several distinct sites in south-eastern Brazil have recorded the exclusive association of *C. cernuum* and *G. bufonia* with *Eufriesea violacea.* For instance, R. L. Dressler collected *E. violacea* males carrying *G. bufonia* pollinaria in Porto Seguro (Bahia State), Santa Leopoldina (Espírito Santo State) and Rio de Janeiro (Rio de Janeiro State) in south-eastern Brazil (R.L. Dressler, unpublished data). Additionally, Singer and Sazima (2004) and Adachi and Silvia Rodrigues Guimarães (2015) documented the exclusive interaction between *G. bufonia* and *E. violacea* in two different locations of the São Paulo State distant at least 500 km from each other. Thereby, *G. bufonia* is only pollinated by male *E. violacea* in at least five sites distant at least 500 km from each other in its geographic range, supporting the assumption that this orchid species is extremely specialized in a single orchid-bee pollinator.

Furthermore, both species have most of their range confined to the area of occurrence of *E. violacea*(Barros et al., 2014; INCT, 2015; Moure et al., 2012) and the authors observed the *E. violacea* and the orchids studied to occur in sympatry at least in the two study sites.

Eufriesea violacea is an orchid-bee occurring in a wide array of forest habitats in the south-eastern portion of South America (Moure et al., 2012; Nemésio, 2009). Both female and male individuals of this species may be important pollinators of the plant assemblage(Rocha-Filho et al., 2012), which they use as sources of food (both sexes) and pheromones (only males) (Eltz et al., 2005). Despite its wide distribution through South America, the occurrence of this species, or at least perfume-collecting males at chemical baits, may be restricted in time, once perfume-collecting males have been collected only in a given period of the rainy season, from November to January (Singer and Sazima, 2004; C.E.P. Nunes, unpublished data). Thus, beyond the cognitive olfactory preferences of *E. violacea,* its restricted occurrence in time would represent another important selective factor in floral traits, especially floral phenology, of species strictly specialised in this orchid-bee, as it is the case of the two species studied.

As mentioned above, *E. violacea* males also pollinate flowers of other orchids sympatric to *G. bufonia* and *C. cernuum*, such as *Cirrhaea* spp., *D. pendula* and *B. harrisoniae.* However, *D. pendula* do not share any floral VOC with the two species studied and the VOC 2-methoxy-4-vinylphenol identified in the floral scent of *D. pendula* as well as the VOCs skatole, vanilin and cineol proved to be attractive to *E. violacea* (C.E.P. Nunes and R.L Dressler unpublished data). The floral scent of *Bifrenaria* and *Cirrhaea* species is also distinct from those of *C. cernuum* and *G. bufonia* and they do not share any of their majoritarian VOCs (G. Gerlach and L.M. Pansarin, unpublished data). Thereby, there are evidences that despite some exclusive compounds may be related to the attraction of *E. violacea* to *C. cernuum* and *G. bufonia*, other sympatric orchid species may use other VOC to enhance pollination by this orchid-bee species. Furthermore, *B. harrisoniae* do not present any apparent floral reward and is also visited by queens of *Bombus brasiliensis* (Apidae: Bombini) and the solitary bee *Xylocopa* (Apidae: Xylocopini), and both bees also carried the pollinaria of this species on their scutellum, so this species may present a more generalist system o pollination by deceit, likely food-deceit (Singer and Koehler, 2004; C.E.P. Nunes unpublished data). *Cirrhaea* species visited by *E. violacea* are also visited and pollinated by males of other orchid-bee species, such as *Euglossa* spp. and *Eulaema nigrita* (Pansarin et al., 2006, 2014).

Catasetum cernuum and *G. bufonia* are phylogenetically allocated in the Cymbidieae tribe of the Epidendroideae subfamily but belong to two well resolved groups (subtribes) of Cymbidieae, respectively, Catasetinae and Stanhopeinae (Givnish et al., 2015; van den Berg et al., 2005). Despite Catasetinae orchids of the genera *Catasetum* Rich. ex Kunth, *Clowesia* Lindl*., Cycnoches* Lindl. and *Mormodes* Lindl. are known to be typically pollinated by perfume-collecting Euglossini (Hymenoptera: Apidae) orchid-bees (Ramírez et al., 2011; van der Pijl and Dodson, 1969; Vogel, 1963), other pollination systems involving other bee taxa and other floral resources are found in the genera *Cyrtopodium* R. Br. and *Grobya* Lindl. in this group (Mickeliunas et al., 2006; Pansarin et al., 2009, 2008). On the other hand, all the known pollinators of Stanhopeinae orchids are male orchid-bees(Gerlach and Dressler, 2003; Pansarin and Amaral, 2009; Whitten et al., 2000). In addition, orchids of the subtribe Stanhopeinae have started their diversification later in relation to Catasetinae, and diversification in Stanhopeinae may have been more closely related to its interaction with male orchid-bees than it may have been in Catasetinae (Freudenstein and Chase, 2015; Givnish et al., 2015). The more diverse pollination systems of Catasetinae and the more specialized of Stanhopeinae, with both groups having their diversification boom much later than most of the diversification of Euglossini bees without evidences of co-divergence(Michel-Salzat et al., 2004; Ramírez et al., 2011) evidence a scenario where different lineages colonized different habitats and fitted their reproductive needs to the existent euglossine or other-bee pollinators. Thereby, the specialization in male orchid-bee pollinators for these two Subtribes as well as for other euglossine-specialized orchids may have been result of adaptive radiation through the occupation of a previously available variety of pollination niches represented by orchid-bees (Givnish et al., 2015; Ramírez et al., 2011, 2007).Whether both the Euglossini-specialized Catasetinae and all Stanhopeinae orchid species derive from ancestral groups pollinated via other means than male-euglossine pollination, likely other bee-pollination systems, is still unknown. Hence, the extreme specialization of *C. cernuum* and *G. bufonia* in the unique pollination niche represented by male *E. violacea* may be a case of evolutionary convergence in an evolutionary irradiation scenario. Such convergent evolution of floral traits have been demonstrated to occur between several Oncidiinae orchids and sympatric oil-offering Malpighiaceae (Papadopulos et al., 2013); however, convergent evolution towards different pollination niches did not prove to be the case for floral scents of South African oil-secreting orchids of the tribe Diseae (Steiner et al., 2011).

To the human vision, some variants of male flowers of *C. cernuum* and the hermaphroditic flowers of *G. bufonia* also share a similar colour pattern, presenting yellowish or greenish flowers covered with vine or red spots (Figs. 1 and 2). Thus, beyond the convergence towards similar olfactory signals, these two species may have also converged towards similar visual cues to enhance recognition and visitation of flowers exclusively by male *E. violacea*. However, studies focusing in the light reflection patterns of these flowers in relation to the visual system of its bee-pollinator are needed to test that assumption.

Using solely male *E. violacea* orchid-bees as pollinators may be advantageous to a given orchid species due to a set of particular traits of the natural history and ecology of this pollinator. *Eufriesea violacea* is a solitary bee that forages and collects perfumes in traplining behaviour. The large size of this bee in relation to most of other sympatric orchid-bees (i.e. *Euglossa* spp.) likely allow relatively larger foraging radius and advantages of trapliner pollinators to plants are well known (Ohashi and Thomson, 2009) and may be particularly advantageous or even essential to sexual reproduction of epiphytic herbs occurring in patchy populations, such as the orchids studied (Trapnell et al., 2004; Winkler et al., 2009). Further, the use of an exclusive pollination niche may also be advantageous to plant species in particular ecological contexts and facilitate plant coexistence(Chen et al., 2009; Pauw, 2013; Schäffler et al., 2015; Soler et al., 2010).

4.3 Conclusion and further work

The similarity of the composition of floral scents of *C. cernuum* and *G. bufonia*, two euglossine-pollinated species derived from distinct Cymbidioid orchid subtribes but with shared unusual floral VOCs, as their pollination by a unique orchid-bee species strongly suggest the floral scent of these species have converged to exploit the exclusive niche of pollination by males *E. violacea*. As a consequence we conclude that the isolated fragrance compound *(E)*-epoxyocimene or a mixture of it with other floral VOCs determines the specific association between the two orchids and their pollinator, male *E. violacea*.

Further studies on floral VOCs and field observations on more orchid species pollinated by *E. violacea*, such as *B. harrisoniae*, *Cirr. dependens* and *Cirr. longiracemosa*, are particularly interesting in order to identify how much these orchids depend on this orchid-bee for pollination and if *(E)*-epoxyocimeneor a similar VOC composes their floral scent. Furthermore, as male *E. violacea* seems to be an important orchid pollinator interacting more or less specifically with at least five sympatric orchid taxa, we think this orchid-bee species is an excellent model for detailed studies and bioassays on the preference for specific substances aiming to better understand how floral VOCs determine plant-pollinator interactions from the individual to the community level.

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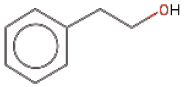
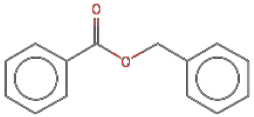
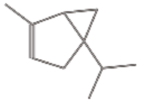
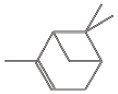
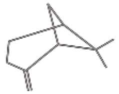
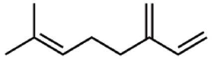
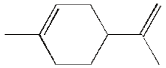
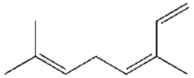
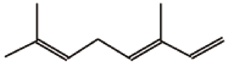
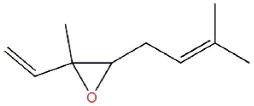
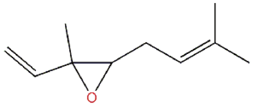
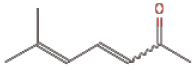
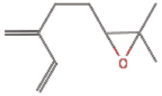
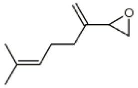
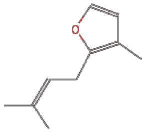
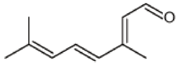
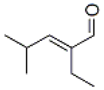
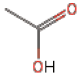
Table

Table 1) Floral volatile organic compounds (VOCs) of *Catasetum cernuum* and *Gongora bufonia.* Relative amounts (%) of odour compounds in headspace fractions of one sample of a female inflorescence of *C. cernuum* and five samples from five samples of *G. bufonia* from at least three distinct localities(- indicates not detected compounds). The compounds are ordered in classes, and listed from lower to higher retention times within each class. Chemical Abstracts Service (CAS) registry numbers and chemical structure are also provided when available.

Compounds	CAS	Structure	*C. cernuum*1 ♀	*C. cernuum* (SJ) ♂	*C. cernuum* (mean ± s.d.)	*G. bufonia* (mean ± s.d.)	*G. bufonia* 1	*G. bufonia* 2	*G. bufonia* (BA)	*G. bufonia* (ES)	*G. bufonia* (SP)
Aromatics (%)			1.5	-	0.75 ± 1.06	0.06 ± 0.086	0.2	-	0.07	0.01	0.056
Monoterpenes (%)			90.1	100	95.05 ± 7	88.53 ± 13.34	95.1	88.7	93.63	65.69	88.528
Other (%)			0.6	-	0.3 ± 0.42	0.05 ± 0.12	-	-	-	0.26	0.052
Total VOCs identified (%)			92.2	100	96.1 ± 5.52	88.63 ± 13.25	99.52	95.3	88.7	93.7	65.96
Total N of VOCs detected			17	3	10 ± 9.9	9.6 ± 3.36	4	11	9	12	12
Non-terpenoids											
4-methylanisole	104-93-8		-	-	-	0.002 ± 0.004	-	-	-	-	0.01
p-cymene	99-87-6		1.5	-	0.75 ± 1.06	-	-	-	-	-	-
phenylethyl alcohol	60-12-8		-	-	-	0.006 ± 0.01	-	-	-	0.03	-
benzyl benzoate	120-51-4		-	-	-	0.05 ± 0.09	-	0.2	-	0.04	-
monoterpenes											
2,6-dimethyl-1,3,5-heptatriene			1.6	-	0.8 ± 1.13	0.004 ± 0.009	0.02	-	-	-	-
α-thujene	2867-05-2		3.9	-	1.95 ±2.76	-	-	-	-	-	-

Compounds	CAS	Structure	*C. cernuum*1 ♀	*C. cernuum* (SJ) ♂	*C. cernuum* (mean ± s.d.)	*G. bufonia* (mean ± s.d.)	*G. bufonia* 1	*G. bufonia* 2	*G. bufonia* (BA)	*G. bufonia* (ES)	*G. bufonia* (SP)
α-pinene	80-56-8		0.6	-	0.3 ± 0.42	0.06 ± 0.09	-	0.1	-	0.2	-
sabinene	3387-41-5		1.3	-	0.65 ± 0.91	-	-	-	-	-	-
β-pinene	127-91-3		0.1	-	0.05 ± 0.07	-	-	-	-	0.03	-
myrcene	123-35-3		18	6.4	12.2 ± 8.2	0.21 ± 0.14	-	0.2	0.4	0.2	0.27
limonene	138-86-3		0.8	-	0.4 ± 0.57	0.12 ± 0.16	-	0.1	0.4	0.1	-
(z)-ocimene	3338-55-4		24	30.2	27.1± 4.38	0.18 ± 0.19	0.5	0.1	0.1	0.2	-
(e)-ocimene	3779-61-1		29	63.4	46.2 ± 24.32	25.73 ± 15.86	44	15	16	42	11.65
(e)-3,4-epoxy-3,7-dimethyl-1,6-octadiene			2.8	-	1.4 ± 1.98	1.09 ± 0.84	-	1	2.2	0.7	1.56

Compounds	CAS	Structure	*C. cernuum*1 ♀	*C. cernuum* (SJ) ♂	*C. cernuum* (mean ± s.d.)	*G. bufonia* (mean ± s.d.)	*G. bufonia* 1	*G. bufonia* 2	*G. bufonia* (BA)	*G. bufonia* (ES)	*G. bufonia* (SP)
(z)-3,4-epoxy-3,7-dimethyl-1,6-octadiene			2.7	-	1.5 ± 1.9	0.41 ± 0.45	-	-	1	0.3	0.76
6-methyl-3,5-heptadien-2-one	1604-28-0		-	-	-	0.20 ± 0.44	-	-	-	-	0.98
6,7-epoxymyrcene			0.5	-	0.25 ± 0.35	0.04 ± 0.09	-	-	0.2	-	-
1,2-epoxymyrcene	153079-54-0		0.5	-	0.25 ± 0.35	-	-	-	-	-	-
3,10-epoxymyrcene			0.3	-	0.15 ± 0.21	-	-	-	-	-	-
rosefuran	15186-51-3		-	-	-	0.14 ± 0.22	-	0.2	-	-	0.5
(e)-epoxyocimene			2.8	-	1.4 ± 1.98	59.13 ± 12.84	55	76.7	68	49.8	46.15
(z)-epoxyocimene			1.2	-	0.6 ± 0.85	0.23 ± 0.19	-	0.2	0.4	0.1	0.45
epoxy rosefuran	92356-06-4		-	-	-	0.83 ± 1.20	-	1.5	-	-	2.63
3,7-dimethyl-octa-2-*(e)*,4-*(e)*,6-trienal	49831-80-3		-	-	-	0.15 ± 0.33	-	-	-	-	0.74

Compounds	CAS	Structure	*C. cernuum*1 ♀	*C. cernuum* (SJ) ♂	*C. cernuum* (mean ± s.d.)	*G. bufonia* (mean ± s.d.)	*G. bufonia* 1	*G. bufonia* 2	*G. bufonia* (BA)	*G. bufonia* (ES)	*G. bufonia* (SP)
4-methyl-ethyl-pent-2-enolid	28419-86-5		-	-	-	0.05 ± 0.11	-	-	-	-	0.26
					Other						
acetic acid			0.6	-	0.3 ± 0.42	-	-	-	-	-	-

Video captions

Video 1) Pollination of the orchid *Gongora bufonia* by the orchid-bee *Eufriesea violacea* through “slip and fall” mechanism. Male *Euf. violacea* pollinating a flower of *G. bufonia* while it collects perfumes and then slips and falls with the back on the stigmatic region of the column (online at https://www.youtube.com/watch?v=BDW23XUGV3s).

Figures

Fig. 1) Plant, inflorescence, flower of *Catasetum cernuum* (A-D) and *Gongora bufonia* (E-G). (A and E) Plant habit of the two orchid species at the Atlantic Forest of Serra do Mar Mountain Range, south-eastern Brazil, (B and E) their inflorescences; (C) female and (D) male flowers of *C. cernuum*; and hermaphroditic flower *G. bufonia.* Arrow indicating the antennae which triggers the pollinaria shooting in *C. cernuum* male flower. Scale bars = 2 cm.

Fig.2) Pollination of *Catasetum cernuum* and *Gongora bufonia* by *Eufriesea violacea* with details on the “slip and fall” mechanism of pollination in *G. bufonia.*(A) Illustration by J.F. de Toledo (Hoehne 1930) showing several male *E. violacea* visiting, two of them carrying pollinarium (arrows)in an individual of *C. cernuum,* a personal observation of F.C. Hoehne(van der Pijl and Dodson 1969). (B-D)Male *E. violacea* pollinating a flower of *G. bufonia* while (B) it collects perfumes and then (C)slips and (D) falls with the back on the stigmatic region of the column; two lip lateral appendages function as handrails, directing the insect as it slips its middle legs down on it during its tumble. Arrows indicate the attached pollinaria of both species in the back of *E. violacea* orchid-bees.

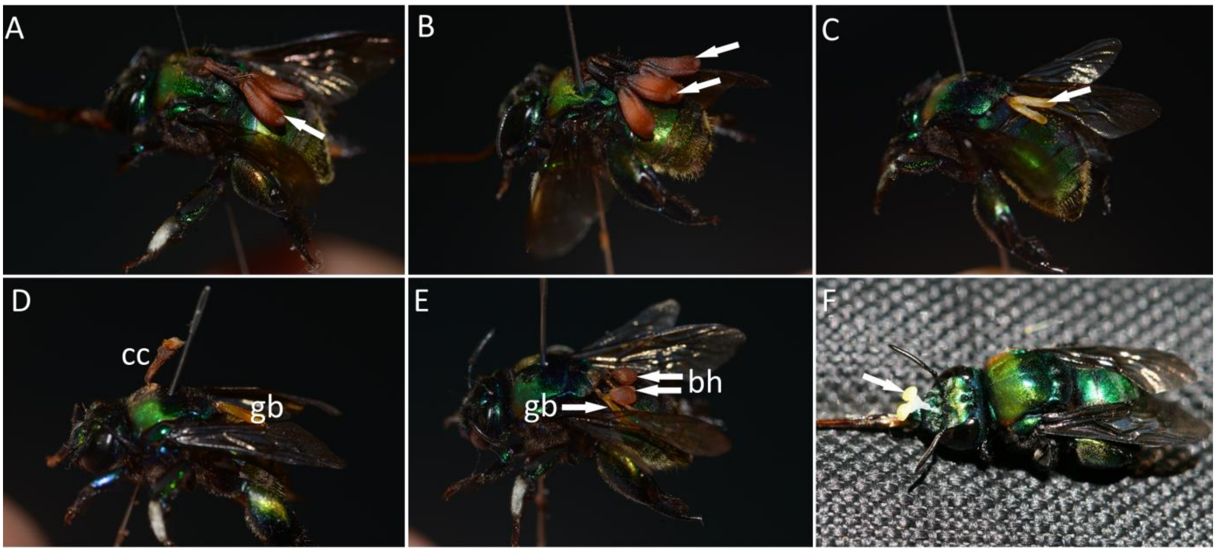


Fig. 3) Male orchid-bees *Eufriesea violacea* captured with pollinaria of four orchid species in south-eastern Brazil. Individuals were collected while carrying (A) a pollinarium of *Catasetum cernuum* (arrow), (B) two pollinaria of *C. cernuum* (arrows),(C) a pollinarium of *Gongora bufonia* (arrow), (D) two pollinaria of *G. bufonia* (gb) and one viscidium plus caudicle of *Catasetum cernuum* (cc), (E) two pollinaria of *Bifrenaria harrisoniae* (arrows, bh) together with one of *G. bufonia* (arrow, gb) and (F) a pollinarium of *Dichaea pendula* (arrow). All the individuals were captured at the natural reserve of Serra do Japi, São Paulo, Brazil, with exception of the one with the pollinarium of *D. pendula* which was captured at Serra do Mar State Park, São Paulo, Brazil.

CONSIDERAÇÕES FINAIS E PERSPECTIVAS

A partir dos resultados da biologia floral, sistema reprodutivo, visitantes e polinizadores podemos concluir que nove das espécies estudadas dependem completamente de polinizadores bióticos para formar frutos e, portanto, para se reproduzir sexualmente. Por outro lado, *Dichaea cogniauxiana* constituiu um caso excepcional de autopolinização: pela interação com o besouro florívoro *Montella* sp. nova e de autopolinização espontânea. Portanto, *D. cogniauxiana* não depende exclusivamente de polinizadores bióticos para formação de frutos. A relativa independência de abelhas como polinizadores nessa espécie pode constituir uma adaptação à escassez de insetos vetores de pólen, uma vez que estas plantas têm sua distribuição restrita às florestas montanas e sub-montanas (acima dos 600 m de altitude), onde abelhas são, em geral, menos abundantes. Entretanto, estudos mais aprofundados e mais observações *in situ* são necessários para confirmar polinizadores responsáveis pela polinização cruzada de *D. cogniauxiana* assim como para determinar os polinizadores de *Pabstia jugosa* e *Promenaea xanthina*, espécies nas quais nenhum polinizador foi observado.

De maneira inédita, este trabalho descreve com detalhes sistema de polinização de berçário (ou *nursery pollination*), em que gorgulhos ativamente polinizam as flores cujos óvulos em desenvolvimento alimentam sua prole. As existências deste sistema de polinização bem como o comportamento de polinização ativa constituem novidades, respectivamente, para a família Orchidaceae e para a ordem Coleoptera. Assim, mais pesquisas são necessárias para elucidar a ecologia e a evolução desse sistema de polinização, assim como sua importância para a reprodução e manutenção das populações de *D. cogniauxiana.* Além disso, este trabalho contribui com o avanço do conhecimento sobre a polinização de orquídeas, em especial da subtribo Zygopetalinae, ao demonstrar a ocorrência de sistemas de polinização por engodo ou exploração sensorial de polinizadores neste grupo, até então conhecido somente por apresentar espécies polinizadas por machos de Euglossina em sua coleta de perfumes.

A análise multivariada das fragrâncias florais permitiu agrupar de maneira consistente (estatisticamente significativa) as espécies estudadas. Entre a maior parte das espécies, os grupos formados correspondem às relações filogenéticas entre seus integrantes e também aos sistemas de polinização. Entretanto, espécies com sistemas de polinização mais restritivos ou especializados diferem das demais e compõem grupos distintos, graças à presença de um ou mais voláteis florais exclusivos em sua fragrância.

Em linhas gerais, estes resultados apontam para a existência de uma predominância e ancestralidade da polinização por abelhas em orquídeas da Mata Atlântica. Especificamente em Zygopetalinae, a polinização pela exploração sensorial de abelhas que buscam alimento apresentou-se como predominante e possivelmente ancestral. Entretanto, ao longo da história evolutiva desta subtribo, os sistemas de polinização teriam passado por uma irradiação adaptativa que teria culminado na diversidade de sistemas hoje encontrada no grupo, que inclui polinização por machos Euglossina, autopolinização e polinização de berçário por besouros Curculionidae.

APÊNDICES

Profa. Dra. Rachel Meneguello
Presidente
Comissão Central de Pós-Graduação
Declaração

As cópias de artigos de minha autoria ou de minha co-autoria, já publicados ou submetidos para publicação em revistas científicas ou anais de congressos sujeitos a arbitragem, que constam da minha Dissertação/Tese de Mestrado/Doutorado, intitulada **"FLORAL SCENT AND POLLINATION ECOLOGY OF ORCHIDS FROM THE ATLANTIC FOREST OF SOUTHEASTERN BRAZIL"**, não infringem os dispositivos da Lei n.° 9.610/98, nem o direito autoral de qualquer editora.

Campinas, 08 de fevereiro de 2016

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Em observância ao **§5º do Artigo 1º da Informação CCPG-UNICAMP/001/15**, referente a Bioética e Biossegurança, declaro que o conteúdo de minha Tese de Doutorado, intitulada “***FLORAL SCENT AND POLLINATION ECOLOGY OF ORCHIDS FROM THE ATLANTIC FOREST OF SOUTHEASTERN BRAZIL***”, desenvolvida no Programa de Pós-Graduação em Biologia Vegetal do Instituto de Biologia da Unicamp, não versa sobre pesquisa envolvendo seres humanos, animais ou temas afetos a Biossegurança.

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