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NÍCOLAS ALBERTO POLIZELLI RICCI

BIOLOGIA REPRODUTIVA E DIVERSIDADE DE FRAGRÂNCIAS FLORAIS DE ORQUÍDEAS *Brasiliorchis*

REPRODUCTIVE BIOLOGY AND FLORAL FRAGRANCES DIVERSITY OF *Brasiliorchis* ORCHIDS

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Tese apresentada ao Instituto de Biologia da Universidade Estadual de Campinas como parte dos requisitos exigidos para a obtenção do título de Doutor em Biologia Vegetal.

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Orientadora: SAMANTHA KOEHLER
Co-orientadores: SILVIA RIBEIRO DE SOUZA
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"Aqui, no entanto, não olhamos para trás por muito tempo. Nós continuamos seguindo em frente, abrindo novas portas e fazendo coisas novas, pois somos curiosos…

E a curiosidade continua nos conduzindo por novos caminhos

Keep moving forward (Siga em frente)"

Walt Disney

Título:

Biologia reprodutiva e diversidade de fragrâncias florais de orquídeas *Brasiliorchis*

RESUMO

A grande diversidade de espécies é decorrente da ação de diferentes barreiras reprodutivas que agem no isolamento reprodutivo, gerando diversidade biológica. Dentre estas barreiras, destacam-se os sistemas de autoincompatibilidade e a variação interespecífica em compostos orgânicos voláteis (COVs) florais. Aqui, estudamos a biologia reprodutiva e a composição química de COVs florais em orquídeas neotropicais *Brasiliorchis.* A motivação para abordarmos estas barreiras reprodutivas é de que a heterogeneidade do bioma da Mata Atlântica representa um desafio adicional para o estudo de grupos ricos em espécies, como Orchidaceae. A variação dos caracteres vegetativos entre os hábitats leva a morfologias sobrepostas e complica a identificação das espécies tradicionalmente reconhecidas. Assim, estudos relacionados à biologia reprodutiva podem contribuir para a abordagem da biossistemática e sua relação com a taxonomia do grupo. Especificamente, para a subtribo Maxillariinae, que contém cerca de 600 espécies neotropicais, o conhecimento sobre sistemas reprodutivos, bem como sobre padrões de plasticidade em COVs florais foi pouco explorado. Portanto, este modelo de estudo é interessante, uma vez que a abordagem dos COVs florais e o sistema reprodutivo dentro do gênero poderia elucidar e contribuir à taxonomia (i.e. se as espécies são aparentadas e difíceis de identificar ou se são espécies-irmãs ou formam complexos de espécies), ecologia e bioconservação destas espécies. A subtribo Maxillariinae apresenta poucos relatos disponíveis sobre plasticidade intra- e interespecífica de COVs florais. Desta forma, a determinação da composição química e o padrão de diversificação dos COVs, em espécies de *Brasiliorchis*, pode resultar em caracteres informativos para identificar linhagens reprodutivamente isoladas. No primeiro capítulo, descrevemos pela primeira vez o sistema reprodutivo de três espécies (*B. phoenicanthera*, *B. picta* e *B. porphyrostele*) e a existência de um sistema de autoincompatibilidade gametofítica para *B. picta* através de estudos anatômicos. No segundo capítulo, descrevemos a composição química de COVs florais em populações distintas de oito espécies do gênero: *B. barbozae*, *B. gracilis*, *B. marginata*, *B. phoenicanthera*, *B. picta*, *B. porphyrostele*, *B. schunkeana* e *B. ubatubana*. Identificamos um total de 238 COVs, que variaram de 34 a 70 por espécie. Os COVs são dominados por categorias distintas de compostos (alcoóis, alcenos, alcanos, aldeídos, monoterpenos e aromáticos). As espécies estudadas apresentaram grande diversidade interespecífica de COVs florais, e outros estudos são necessários para verificar a variação intraespecífica de COVs florais em *Brasiliorchis*. Processos de seleção natural e deriva podem ter atuado para a diversificação de fragrâncias florais e contribuído para o isolamento reprodutivo de espécies.

Palavras-chave: anatomia floral, autoincompatibilidade, compostos orgânicos voláteis, Epidendroideae, ecologia química, GSI, Mata Atlântica, Maxillariinae, Orchidaceae, sistema reprodutivo.

Reproductive biology and floral fragrances diversity of *Brasiliorchis* orchids

ABSTRACT

Species diversity is a product of different reproductive barriers acting in isolation of evolutionary lineages. Among these barriers, there are self-incompatibility systems and interspecific variation of floral volatile organic compounds (VOCs). Here, we study the reproductive biology and chemical composition of floral VOCs in Neotropical *Brasiliorchis* orchids. The motivation for approaching these reproductive barriers is that the heterogeneity of the Atlantic Forest biome represents an additional challenge for the study of species-rich groups, such as Orchidaceae. The variation of vegetative characters between habitats leads to overlapping morphologies and complicates the identification of traditionally recognized species. Thus, studies related to reproductive biology can contribute to the approach of biosystematics and its relationship with the taxonomy of the group. Specifically, for the subtribe Maxillariinae, which contains about 600 Neotropical species, knowledge about reproductive systems as well as patterns of plasticity in floral VOCs has been little explored. Therefore, this model is interesting, since the approach of floral VOCs and the reproductive system within the genus could elucidate and contribute to the taxonomy (i.e. whether the species are related and difficult to identify or whether they are sister species or form species complexes), ecology and bioconservation. Maxillariinae has few available reports on intra- and interspecific plasticity of floral VOCs. In this way, the determination of the chemical composition and the diversification pattern of the VOCs, in *Brasiliorchis* species, can result in informative characters to identify reproductively isolated lineages. In the first chapter, we describe, for the first time, the reproductive system of three species (*B. phoenicanthera*, *B. picta* and *B. porphyrostele*) and the existence of a gametophytic self-incompatibility system for *B. picta* through anatomical studies. In the second chapter, we describe the chemical composition of floral VOCs in different populations of eight species of the genus: *B. barbozae*, *B. gracilis*, *B. marginata*, *B. phoenicanthera, B. picta, B. porphyrostele, B. schunkeana and B. ubatubana*. We identified a total of 238 VOCs, which ranged from 34 to 70 per species. VOCs are dominated by distinct categories of compounds (alcohols, alkenes, alkanes, aldehydes, monoterpenes and aromatics). The studied species showed great interspecific diversity of floral VOCs, and more studies are very important to verify intraspecific diversity of floral VOCs in *Brasiliorchis*. Natural selection and drift processes may have acted to diversify floral fragrances and contributed to the reproductive isolation of species.

Keywords: floral anatomy, self-incompatibility, volatile organic compounds, Epidendroideae, chemical ecology, GSI, Atlantic Forest, Maxillariinae, Orchidaceae, reproductive system.

SUMÁRIO

INTRODUÇÃO GERAL

A IMPORTÂNCIA DA FAMÍLIA ORCHIDACEAE: POTENCIAIS ORNAMENTAL, ECONÔMICO E BIOCONSERVACIONISTA

As plantas ornamentais apresentam importância econômica significativa, assim como a contribuição na Ecologia e Botânica (Chugh et al., 2009). Dentre as que mais contribuem com o âmbito econômico estão as orquídeas. De acordo com Schoenmaker (2017), a comercialização de orquídeas é intensamente relevante e a tendência é que continue em ascensão. O Congresso Brasileiro de Cultura de Tecidos de Plantas (CBCTP) divulgou, no ano de 2012, dados que mostram a crescente elaboração de trabalhos que fazem uso de plantas e flores, realizando pesquisas científicas e tecnológicas, sendo que os números mostram que de todos os trabalhos abordados, 31,23% são referentes às orquídeas. Com tantos estudos sendo desenvolvidos, é reafirmada a valorização e o interesse da família Orchidaceae, mundialmente (Boscolo et al., 2010; Souza e Junghans, 2013). As orquídeas são cultivadas sob diferentes fatores (temperatura, luz, umidade, nutrientes, etc.), que são estudados e adequados para cada espécie/gênero de orquídea (Minamiguchi e Machado-Neto, 2007). A importância das orquídeas contribuiu para o desenvolvimento de técnicas biotecnológicas, como a cultura de células e tecidos vegetais, uma vez que a micropropagação também auxilia na obtenção de plantas saudáveis em menor tempo (Costa et al., 2013), e consequemente a técnica diminui os gastos resultantes de metodologias que demandam maior tempo de execução. No Brasil, desde a década de 1970, é realizada a técnica de micropropagação *in vitro* de orquídeas, com a intenção de elevar a produção de mudas, salvando inúmeras espécies da extinção (Stancato et al., 2001).

As orquídeas encantam as pessoas pela beleza exótica que é possível graças à enorme diversidade existente. As orquídeas são plantas ornamentais imprescindíveis e de grande interesse botânico e econômico (Cottrell, 2000), visto que seu cultivo comercial iniciou-se há mais de 150 anos, no continente Europeu. Alguns casos famosos são os híbridos de espécies de *Vanilla*, cujos frutos dão origem à baunilha natural utilizada na culinária e indústria alimentícia, sendo este gênero originário da Guatemala, México, Brasil, Madagascar, entre outros (May et al., 2006). O uso de orquídeas em áreas, que não a ornamental, outrossim foi relatado na cosmética e na medicina natural. A pesquisa ocorre, inicialmente, com a extração e a aplicação de óleos essenciais de orquídeas, e culmina com o conhecimento dos seus efeitos nos diferentes sistemas do corpo humano. Isso ocorre por meio da elaboração de óleos

corporais, hidratantes, sabonetes, cremes antienvelhecimento, perfumaria e outros, uma vez que são apontadas propriedades antioxidantes, imunológicas e antitumorais. No livro de Dioscórides, escrito 100 anos d.C., há relatos de aproximadamente 600 plantas medicinais, e dentre elas, as orquídeas são localizadas como importantes na propulsão da fertilidade e virilidade. Logo, eram utilizadas para fabricação de bebidas afrodisíacas, como a obtida a partir do sistema radicular de *Orchis mascula* L. (Sahlep) (Ventura, 2002). Há relatos do uso de orquídeas na indústria alimentícia, por se assemelharem a produtos como aromatizantes, amidos ou bebidas e na geração de compostos químicos (ácidos gálicos, taninos, resinas, glucosídeos e quinonas fenantrenos) (Ventura, 2002). Segundo estudos, as orquídeas possuem características que as tornam úteis ao tratamento de dores de cabeça, artrite, problemas digestivos, anticoncepcional e cicatrizantes (*Cymbidium madidum* Lindl.) (Ventura, 2002; Petrovska, 2012). Na busca crescente por estabilidade emocional e bloqueios de energias negativas, também são empregados os óleos essenciais extraídos de orquídeas do gênero *Phalaenopsis*, de forma a amenizar os sintomas da menopausa, inflamações nas articulações e herpes (Ventura, 2002; Petrovska, 2012).

No cenário econômico, no Brasil, as orquídeas estimulam o mercado através de números significativos: entre as espécies de elevado valor comercial, encontram-se a espécie *Cattleya walkeriana* Gardner, conhecida também por feiticeira, que chega a valer R$2.500,00. Há variedades que atingem valores de US$5.000,00, no mercado internacional (Rodrigues, 2010). Segundo Junqueira e Peetz (2008), o consumo e a geração de plantas ornamentais, no Brasil, cresceu acompanhando o mercado internacional, e tal compartimento econômico passou a empregar 5.152 produtores, com um total de 8.423 hectares de área. A expansão foi decorrente da movimentação econômica do mercado anual que, em 2007, registrou US$1,3 bilhão (Junqueira e Peetz, 2008). Em 2013, os dados demonstraram um aumento no número de produtores, que passou a ser 7.800, com um valor total de área de 13.468 hectares. A região Sudeste prosseguiu com a maior concentração de produtores (53,3%), seguido da região Sul (28,6%) (Junqueira e Peetz, 2014). Dentre as orquídeas com maior prestígio comercial, encontram-se as dos gêneros *Oncidium*, *Dendrobium*, *Cymbidium* e *Phalaenopsis*, e juntas, elas compõem majoritariamente o mercado de mudas em vasos. A produção de espécies de *Phalaenopsis* elevou-se também em outros países como o Japão, Alemanha, China, Holanda e Tailândia (Schoenmaker, 2017), ao longo da primeira década do século XXI.

Sob o contexto da bioconservação, o bioma da Mata Atlântica, considerado um *hotspot*, com perda de metade de sua área de vegetação original (de 14% para 7%), é

provavelmente um dos mais devastados e ameaçados do Brasil, juntamente com o Cerrado, sofrendo um ritmo de modificações que está entre os mais rápidos observados. Portanto, a necessidade de ações que visem à conservação é de grande urgência (Dean, 1996; Young, 2005). O processo de devastação foi iniciado no período colonial brasileiro, sofrendo os impactos dos subsequentes ciclos econômicos ao longo da linha histórica, principalmente o intenso processo de urbanização e crescimento desenfreado dos núcleos urbanos. O extrativismo, bastante praticado na época do Brasil colonial, perdurou até o século XX, a partir do comércio ilegal de peles e penas de animais, assim como plantas ornamentais, tais como orquídeas e bromélias, destinadas à Europa (Dean, 1996; Young, 2005). A remoção em larga escala de madeira de Lei, neste período, também trouxe grande impacto nas florestas primárias, que após utilizadas eram queimadas, vendidas ou abandonadas (Dean, 1996). Hoje, cerca de 120 milhões de brasileiros vivem no território correspondente à área original da Mata Atlântica, onde situam-se as maiores cidades do Brasil, bem como os principais polos industriais, sendo essas regiões responsáveis por um percentual de 80% do PIB brasileiro (Pinto e Brito, 2005; Drummond, 2008). Os principais fatores que contribuíram para a supressão da vegetação foram a exploração madeireira, as queimadas, a conversão de campos naturais em pastagens com espécies exóticas de gramíneas, abertura de ferrovias e rodovias, crescimento populacional, além das extensas áreas de monoculturas de soja, café e cana-de-açúcar (Dean, 1996; Young, 2005; Drummond, 2008).

O inventário florístico florestal de Santa Catarina realizado por Vibrans et al. (2013), mostrou que pelo menos 328 espécies da flora nativa do Estado são utilizadas sob as mais diversas finalidades: madeira, energia, medicina e alimento, e possuem vital importância para as populações adjacentes. Das mais de 20 mil espécies de plantas presentes na Mata Atlântica, aproximadamente 300 constam na lista oficial de espécies ameaçadas, entre elas o palmito-juçara (*Euterpe edulis* Mart.), a araucária (*Araucaria angustifolia* (Bertol.) Kuntze) e várias orquídeas e bromélias. Muitas destas espécies são fundamentais na dinâmica de seus respectivos ecossistemas, pois são consideradas espécies-chave, ou seja, sua extinção poderia causar a perda de várias outras espécies que dependem diretamente delas para sua sobrevivência (Campanili e Schaffer, 2010). Com a finalidade de manter as espécies-chave, algumas soluções seriam os corredores ecológicos e a restauração das áreas degradadas com o plantio de árvores nativas de rápido crescimento para melhorar as características da paisagem e manter as relações ecológicas no bioma. Por fim, outro ponto preocupante é a diminuição ou perda de fluxo gênico

entre as espécies, em decorrência de atividades supressoras humanas, que interfem diretamente na ação de barreiras reprodutivas e na diversidade biológica (Campanili e Schaffer, 2010).

Com aproximadamente 25.000 espécies descritas, Orchidaceae é a maior família de Angiospermas (de Melo et al., 2011; Govaerts et al., 2017). A grande diversidade de espécies pode ser decorrente de diferentes barreiras reprodutivas que agem no isolamento reprodutivo e geram diversidade biológica (Rieseberg e Willis, 2007). Dentre estas barreiras, destacam-se os Sistemas de Autoincompatibilidade (SI - Self-Incompatibility Systems) e a variação interespecífica em compostos orgânicos voláteis (VOCs - Volatile Organic Compounds) florais entre espécies aparentadas de orquídeas. Analisando como o bioma da Mata Atlântica foi amplamente exaurido, tais tópicos foram selecionados para o presente estudo, visando a importância destas barreiras em espécies endêmicas de biomas altamente modificados por atividades antrópicas.

O QUE SÃO SISTEMAS DE AUTOINCOMPATIBILIDADE?

A Autoincompatibilidade é uma importante barreira pré-zigótica/pós-polinização - Autoincompatibilidade Esporofítica (SSI - Sporophytic Self-Incompatibility) e Autoincompatibilidade Gametofítica (GSI - Gametophytic Self-Incompatibility) e pré e pós-zigótica/pós-polinização - Autoincompatibilidade de Ação Tardia (LSI – Late-acting Self-Incompatibility), nas Angiospermas. Esta barreira controlada geneticamente tem por função evitar a autofecundação e, consequentemente, a endogamia, na progênie, culminando no aumento da variabilidade genética (Williams, 2008). Em todos os Sistemas de Autoincompatibilidade, o reconhecimento do próprio pólen é baseado em interações específicas entre alelos, por meio dos produtos expressos do estigma, estilete e ovário. Tais interações desencadeiam uma resposta celular, inibindo a germinação dos grãos de pólen e o desenvolvimento dos tubos polínicos (Mulcahy et al., 1996; Spielman e Scott, 2008).

Os estudos de Sistemas de Autoincompatibilidade surgiram no início do século XX (Stout, 1917) devido à redução de produtividade e dependência da presença de polinizadores em culturas agrícolas. Nas décadas posteriores, suas consequências para o sucesso reprodutivo foram estabelecidos em estudos pioneiros (Prell, 1921; East e Mangelsdorf, 1925; Filzer, 1926; Lehmann, 1926; East, 1940; Brewbaker, 1957). Particularmente, os Sistemas de Autoincompatibilidade são classificados em três tipos, de acordo com a ocorrência da reação

como resposta ao genótipo do pólen (De Nattencourt, 1977; Richards, 1997; Bittencourt et al., 2003; Gibbs, 2014; Oliveira e Maruyama, 2014).

A GSI (Figura 1A) foi descrita inicialmente em espécies do gênero *Nicotiana* (Solanaceae) e definida por um único gene (gene-*S* - *Sterility*), com vários alelos sem dominância (De Nettancourt, 1977; Gibbs, 1990; Oliveira e Maruyama, 2014). O gene-*S* controla a expressão de síntese de moléculas proteicas, tanto no pistilo, quanto nos tubos polínicos, de maneira que a presença de alelos iguais leva a uma reação de autoincompatibilidade. Heslop-Harrison e Shivanna (1977) e Oliveira e Gibbs (1994) mostraram que este sistema é amplamente distribuído nas Angiospermas, relacionado às plantas com estigma úmido (i.e., estigma altamente secretor), no qual os grãos de pólen hidratam e germinam imediatamente. Assim, a autoincompatibilidade ocorre ao longo do estilete, causando, em geral, degradação ou rompimento do crescimento precoce dos tubos polínicos, além de deposições irregulares de calose (Richards, 1997; Rea e Nasrallah, 2008; Gibbs, 2014). O produto do gene-*S*, em plantas com GSI, é expresso na camada interna do grão de pólen (intina), o qual está relacionado ao genoma haplóide do grão de pólen (Griffiths et al., 2009).

Nos anos 50, estudos realizados por Bateman (1952, 1954) mostraram que algumas espécies de plantas apresentavam um mecanismo de autoincompatibilidade distinto da GSI, denominado SSI. Nestas espécies, o controle da autoincompatibilidade também envolve um único lócus com vários alelos diferentes, igual ao sistema GSI, mas no SSI há uma relação de dominância entre os alelos, em que apenas os alelos dominantes são expressos. Ou seja, independente do genótipo haplóide do pólen, as reações que levam à autoincompatibilidade são determinadas pelo genótipo do esporófito (Figura 1B). Por causa da dominância entre os alelos no sistema SSI, as plantas que portam um alelo dominante, além de autoincompatíveis, também são interincompatíveis (i.e., incompatibilidade entre indivíduos). Ademais, diferente do sistema GSI, a dominância entre alelos não permite que ocorra compatibilidade parcial entre indivíduos (Oliveira e Maruyama, 2014; Gibbs, 2014). Plantas com o sistema SSI apresentam costumeiramente o estigma papiloso seco (i.e., estigma não secretor) e estão restritas, principalmente, às famílias Asteraceae, Brassicaceae, Betulaceae, Caryophyllaceae, Convolvulaceae e Polemoniaceae (Richards, 1997; De Nettancourt, esta desencadeia a falha de germinação dos grãos de pólen. Como consequência, tubos polínicos incompatíveis raramente crescem no pistilo e a reação de autoincompatibilidade ocorre na superfície estigmática (Oliveira e Gibbs, 1994). O produto do gene-*S*, no sistema SSI, é uma substância derivada de células da antera, depositadas na camada externa dos grãos de pólen (exina) durante o processo de gametogênese masculina (De Nettancourt, 1999). Comumente, plantas com tal sistema

apresentam pólen tricelular e exina esculturada, esta última associada à deposição de substâncias e interação entre alelos, que desencadeiam a autoincompatibilidade.

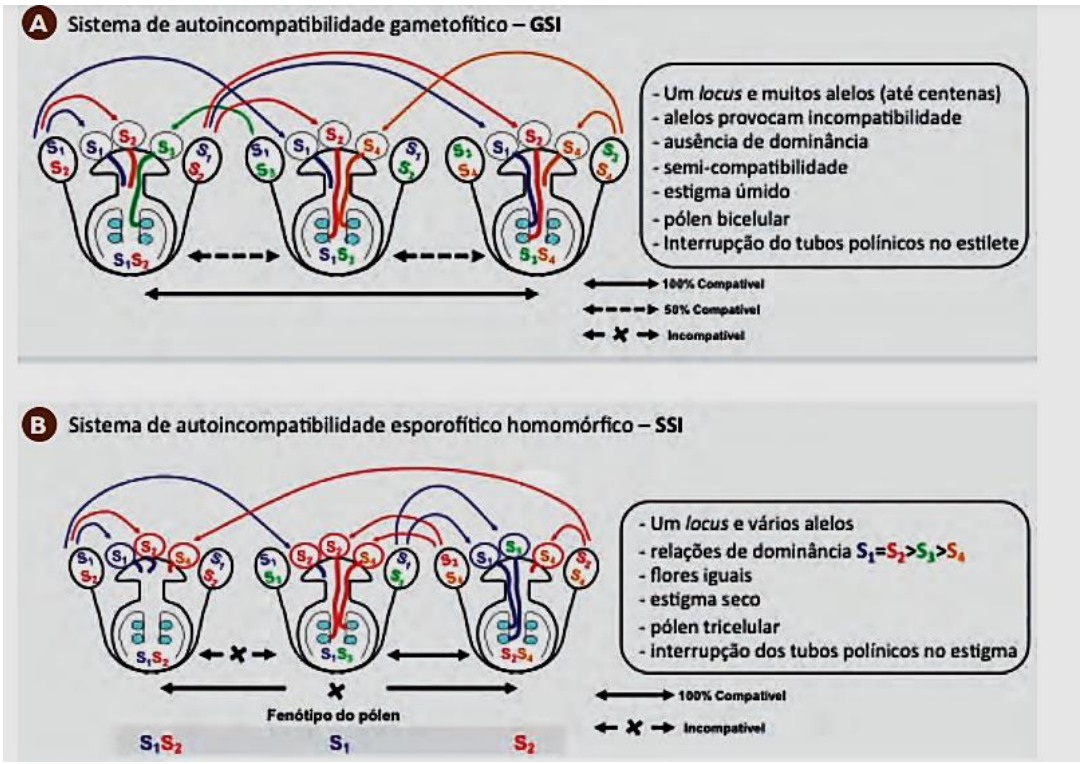

FIGURA 1. Características imprescindíveis aos sistemas clássicos de autoincompatibilidade em plantas: **(A)** Sistema de Autoincompatibilidade Gametofítico (GSI): cruzamentos possíveis entre indivíduos com genótipos diferentes e o comportamento de crescimento de tubos polínicos nos pistilos. São indicados ainda os resultados em termos de compatibilidade e, no quadro, as principais características do sistema. **(B)** Sistema de Autoincompatibilidade Esporofítico Homomorfico (SSI): esquemas semelhantes, mostrando os resultados dos cruzamentos possíveis, influenciados pelas relações de dominância entre alelos. São mostrados os resultados em termos de compatibilidade e o quadro detalha as características do sistema (esquemas extraídos de Richards, 1986; Gibbs, 1990).

A morfologia do crescimento dos tubos polínicos e as diferenças morfológicas/anatômicas observadas com relação ao local de reação da autoincompatibilidade foram utilizados como indicadores dos sistemas GSI ou SSI para zonas temperadas, mas quando estudos foram empregados às espécies tropicais, resultados distintos foram obtidos (Oliveira e Maruyama, 2014). Um exemplo de estudo em zona tropical foi desenvolvido por Cope (1962), com *Theobroma cacao* L. O autor sugeriu a existência de um sistema SSI, com indivíduos parcialmente compatíveis e um número reduzido de alelos. No entanto, experimentos utilizando microscopia de fluorescência mostraram que os tubos polínicos cresciam até os óvulos e havia fertilização parcial (Cope, 1962; Ford e Wilkinson, 2012). A autoincompatibilidade, neste caso, é resultante da ineficácia de fusão dos núcleos polares para a formação da célula primária do endosperma. Desta forma, a LSI foi descrita após os demais sistemas, e a reação ocorre no

ovário, aparentemente sem afetar o crescimento dos tubos polínicos (De Nettancourt, 1977, 1984; Seavey e Bawa, 1986). A reação da LSI pode ocorrer em diferentes estágios: antes do óvulo ser fecundado, antes da formação do zigoto, após a formação do zigoto e por inibição no óvulo (Seavey e Bawa, 1986; Bittencourt et al., 2003; Gibbs, 2014). Seavey e Bawa (1986) e Oliveira (1998), constataram que estes fenômenos são comuns em plantas com flores pequenas em relação aos frutos, nas quais o aborto pós-polinização constitui um mecanismo de seleção de baixo custo. Sistemas GSI e SSI, por outro lado, são comuns em flores grandes e custosas, nas quais a autoincompatibilidade aumenta a ocorrência de polinização cruzada e redução dos custos associados à depressão endogâmica. Entretanto, a atuação da SSI na superfície estigmática confere maior eficácia energética e reprodutiva do que a GSI e o LSI pré ou pós-zigótico (Rea e Nasrallah, 2008).

QUAIS SÃO OS SISTEMAS DE AUTOINCOMPATIBILIDADE EM ORCHIDACEAE?

Embora seja uma das mais diversas famílias de Angiospermas, o conhecimento sobre os Sistemas de Autoincompatibilidade em orquídeas é restrito a poucas espécies. Niu et al. (2017) destacam que estes sistemas são raros na família, estimando sua ocorrência em apenas 10% das espécies já estudadas quanto ao sistema reprodutivo. Outros estudos sugerem que a autoincompatibilidade está restrita a subtribos específicas em três subfamílias de Orchidaceae: subtribo Cypripediinae (subfamília Cypripedioideae) (Pedersen et al., 2012); subtribo Orchidinae (subfamília Orchidoideae) (Dieringer, 1982); e nas subtribos Aerangidinae (Agnew, 1986), Aeridinae (Agnew, 1986; Huda e Wilcock, 2008; Cai et al., 2015), Angraecinae (Agnew, 1986), Coelogyninae (Cheng et al., 2009; Liu et al., 2013), Dendrobiinae (Johansen, 1990; Huda e Wilcock, 2008; Pinheiro et al., 2015), Laeliinae (East, 1940; Ackerman, 1989), Limodorinae (East, 1940), Lycastinae (Hietz et al., 2006), Malaxidinae (Whigham e O'Neil, 1991; Aragón e Ackerman, 2001; Oh et al., 2001), Oncidiinae (East, 1940; Ackerman e Montero-Oliver, 1985; Parra-Tabla et al., 2000; Ospina-Calderón et al., 2015; Aguiar e Pansarin, 2019), Pleurothallidinae (Christensen, 1992; Borba et al., 2001; Tremblay et al., 2006; Gontijo et al., 2010; Borba et al., 2011; de Melo et al., 2011; Barbosa et al., 2013; Duarte et al., 2020) e Sarcanthinae (Agnew, 1986) (subfamília Epidendroideae) (Tabela S1, Capítulo 1).

A subfamília Epidendroideae contém 80% das espécies da família e apresenta o maior número de clados com espécies autoincompatíveis descritas. Espécies autoincompatíveis

ocorrem principalmente nas subtribos Dendrobiinae, Oncidiinae e Pleurothallidinae (Tabela S1). Na subtribo Maxillariinae, uma das mais diversas na região neotropical, com aproximadamente 600 espécies reconhecidas (Govaerts et al., 2005; Whitten et al., 2007), pouco se conhece sobre a biologia reprodutiva destas espécies (Singer, 2003), apesar da alta diversidade de estratégias reprodutivas (e.g., pseudocópula, imitação de locais de nidificação e abrigo, exploração do comportamento dos polinizadores) (Jersáková et al., 2006). Apenas dois estudos sugeriram espécies autoincompatíveis na subtribo Maxillariinae e foram publicados por East (1940) e Hietz et al. (2006), que incluíam *Lycaste cruenta* (Lindl.) Lindl. (syn. *Maxillaria skinneri* Lindl.) e *Lycaste aromatica* (Graham) Lindl., respectivamente.

O QUE SÃO OS COMPOSTOS ORGÂNICOS VOLÁTEIS FLORAIS E QUAL A FUNÇÃO DELES NO SUCESSO REPRODUTIVO?

Para assegurar o sucesso reprodutivo, as Angiospermas, comumente exploram o comportamento animal de busca por alimentos provenientes dos recursos florais (Dafni, 1984; Majetic et al., 2009). No caso da interação planta-animal, sinais atrativos, como visuais (cor, forma e simetria) e olfativo (odor) anunciam a presença de recursos, como pólen, néctar, óleos, resina ou ceras (Jersáková et al., 2009). Como recursos florais (e.g., resina, óleo floral e fragrância) são energeticamente muito custosos, muitas angiospermas desenvolveram distintas estratégias de polinização por engodo, principalmente as espécies das famílias Aristolochiaceae, Apocynaceae, Araceae e Orchidaceae (Jersáková et al., 2009). Neste caso, as plantas mimetizam caracteres típicos de estruturas que ofertam recursos florais, seja pela morfologia, cor ou até mesmo fragrâncias (COVs) (Galizia et al., 2005; Jersáková et al., 2006; Jersáková et al, 2012).

Compostos orgânicos voláteis (COVs) são metabólitos secundários liberados pelas flores e são constituídos, principalmente, de moléculas químicas de baixa massa molecular, incluindo, terpenoides (mono e sesquiterpenos), aromáticos benzenoides e derivados de ácidos graxos (Dudareva et al., 2013). Os COVs são uma característica importante dentro de plantas ornamentais e também é um fator de grande magnitude no processo evolutivo, pois estes sinais olfativos atraem polinizadores, visitantes, animais dispersores de sementes, oportunistas como pilhadores de recursos florais e até mesmo herbívoros (Schiestl, 2015; Borghi et al., 2017). Por exemplo, em orquídeas *Chiloglottis*, a presença de voláteis específicos atrai polinizadores especializados, o que pode levar ao isolamento reprodutivo e à evolução (Peakall e Whitehead, 2014). No entanto, os COVs também têm a função biológica de conferirem proteção à planta,

como defesa contra pragas, herbívoros e microrganismos colonizadores (Holopainen e Gershenzon, 2010; Ortega et al., 2016), que geralmente estão correlacionados com a evolução nas rotas biossintéticas de produção de COVs (Schiestl, 2010).

Orchidaceae apresenta a maior concentração de espécies com sistemas de polinização por engodo (Van der Pijl e Dodson, 1966; Ackerman, 1986; Jersáková et al., 2006). Estima-se que 6.500 espécies de orquídeas, distribuídas em 47 gêneros, possuem sistemas de polinização por engodo generalizado (não-sexual). Este mecanismo, encontrado em um terço de todas as orquídeas, é a estratégia reprodutiva de engodo mais comum (Jersáková et al., 2006; Renner, 2006). Em contrapartida, apenas ca. 600 espécies apresentam sistema de polinização por pseudocópula, caracterizado pela simulação de feromônios sexuais utilizados pelos insetos durante o voo nupcial (Jersáková et al., 2006). Assim, os machos são atraídos pelas flores destas espécies de orquídeas em busca de fêmeas, efetuando a polinização (Vereecken, 2009). Este caso de polinização, que é exclusivo da família (Van der Cingel, 2001; Schiestl, 2005), atinge altos graus de especificidade, e segundo Schiestl e Cozzolino (2008), pode ter evoluído a partir de sinais florais utilizados por insetos durante o processo de cópula.

Ao longo do processo evolutivo, os polinizadores estão constantemente adaptando seu comportamento durante o forrageio, fator este que levaria ao reconhecimento de flores sem recursos (Smithson e Macnair, 1997; Juillet et al., 2011). Por este motivo, é esperado que plantas com engodo generalizado exibam grande plasticidade, principalmente na cor e/ou COVs florais, de forma a retardar o aprendizado de polinizadores em relação à ausência de recursos florais (Moya e Ackerman, 1993; Jersáková et al., 2009). Portanto, a capacidade das plantas de desenvolver novas vias ou modificar vias existentes para sintetizar fragrâncias florais distintas é crucial para a estabilidade evolutiva da polinização por engodo generalizado (Schiestl e Schlüter, 2009). De acordo com esta premissa, espera-se encontrar uma grande variação na composição de COVs florais entre indivíduos de diferentes espécies polinizados por engodo generalizado (Moya e Ackerman, 1993; Jersáková et al., 2009). No entanto, neste caso, a variação intraespecífica de COVs florais deve ser menor do que a variação interespecífica (Dobson et al., 1997; Byers et al., 2014). Tal hipótese já foi corroborada para a variação morfológica de flores de engodo, pois os polinizadores não associam a morfologia floral à ausência de recursos (Smithson e Macnair, 1997).

Estudos com Orchidaceae que consideraram a plasticidade da composição de COVs florais não observaram maior sucesso reprodutivo em espécies com maior variação das fragrâncias florais (Juillet e Scopece, 2010; Juillet et al., 2011). Em conclusão, os autores sugerem que a alta variabilidade da composição química nas fragrâncias das flores não evita o

aprendizado por parte dos polinizadores, como frequentemente é proposto, mas pode retardar o processo cognitivo dos polinizadores. Sobre isso, o estudo de Vereecken e Schiestl (2008) verificou que a preferência de indivíduos da abelha *Colletes cunicularius* (Linnaeus, 1758) por fragrâncias distintas desencadeia uma pressão seletiva sobre o bouquet de aromas da orquídea *Ophrys sphegodes* Mill. (syn. *Ophrys exaltata* Ten.), uma vez que a distinção de fragrâncias florais de um indivíduo a outro atrairia mais visitantes florais do que indivíduos com menor variação. Este estudo traz um recorte de como a variação de fragrâncias florais em espécies polinizadas por engodo pode ser vantajosa. A partir desta premissa, mais estudos comparativos, considerando a composição de COVs em grupos de orquídeas morfologicamente similares e polinizados por engodo, são necessários para esclarecer esta questão. Para tanto, o presente estudo busca compreender a plasticidade de COVs em orquídeas de engodo, morfologicamente semelhantes e simpátricas do gênero *Brasiliorchis.*

POR QUE ESTUDAR SISTEMA DE AUTOINCOMPATIBILIDADE E COVs FLORAIS EM *Brasiliorchis*?

Brasiliorchis é um dos gêneros de orquídeas brasileiras taxonomicamente mais complexos. A ausência de caracteres morfológicos diagnósticos claros dificulta o desenvolvimento de estudos em outras áreas do conhecimento biológico, além de ser um obstáculo para a comercialização de suas espécies, que são de grande valor ornamental, bem como para elaboração de medidas para conservação e combate à biopirataria (comércio ilegal) (Flores-Palácios e Valencia-Díaz, 2007; Swarts e Dixon, 2009).

O estudo filogenético de Whitten et al. (2007) recircunscreveu gêneros para subtribo Maxillariinae (tribo Cymbidieae) de acordo com relações filogenéticas baseadas em sequências de DNA nuclear e plastidiais. Dentre os gêneros segregados está *Brasiliorchis* R. Singer, S. Koehler & Carnevali, que é distinguido de outras orquídeas da subtribo Maxillariinae pela presença de pseudobulbos bifoliados, sulcados a estriados, folhas conduplicadas, longas e estreitas, flores com fragrância adocicada semelhante a mel, antese de longa-duração, sem recursos florais e com polinários geralmente desprovidos de estipes (Singer et al., 2007). *Brasiliorchis* é um gênero que ocorre na Mata Atlântica e na vegetação circundante, principalmente no Brasil, dos Estados do Rio Grande do Sul à Bahia e no Pernambuco, estendendo-se até Misiones, Argentina (Hoehne, 1953; Pabst e Dungs, 1977; Waechter, 1996; Toscano de Brito e Cribb, 2005; Singer et al.,

2007; Barros et al., 2015). Atualmente, 15 espécies são reconhecidas (Singer et al. 2007) (Tabela 1).

TABELA 1. Distribuição das 15 espécies de *Brasiliorchis*, atualmente reconhecidas, no bioma da Mata Atlântica. Regiões Brasileiras: CO = Centro-Oeste, NE = Nordeste, SE = Sudeste e S = Sul. Estados Brasileiros: BA = Bahia, ES = Espírito Santo, MG = Minas Gerais, MS = Mato Grosso do Sul, PE = Pernambuco, PR = Paraná, RJ = Rio de Janeiro, RS = Rio Grande do Sul, SC = Santa Catarina e SP = São Paulo.

Regiões Brasileiras	**CO**	**NE**		**SE**				**S**		
Estados Brasileiros	**MS**	**BA**	**PE**	**ES**	**MG**	**RJ**	**SP**	**PR**	**RS**	**SC**
Espécies										
B. barbozae (Loefgr.) R.B.Singer et al.		X				X	X			
B. chrysantha (Barb.Rodr.) R.B.Singer et al.		X		X	X	X	X	X	X	X
B. consanguinea (Klotzsch) R.B.Singer et al.				X	X	X	X	X		
B. gracilis (Lodd.) R.B.Singer et al.		X	X	X	X	X	X	X	X	X
B. heismanniana (Barb.Rodr.) R.B.Singer et al.				X						
B. kautskyi (Pabst) R.B.Singer et al.			X	X						
B. marginata (Lindl.) R.B.Singer et al.		X		X	X	X	X	X	X	X
B. monantha (Barb.Rodr.) Campacci				X	X		X	X		X
B. moutinhoi (Pabst) F.Barros & L.Guimarães						X				
B. phoenicanthera (Barb.Rodr.) R.B.Singer et al.				X	X		X	X		X
B. picta (Hook.) R.B.Singer et al.	X			X	X		X	X	X	X
B. polyantha (Barb.Rodr.) R.B.Singer et al.										
B. porphyrostele (Rchb.f.) R.B.Singer et al.					X		X	X	X	X
B. schunkeana (Campacci & Kautsky) R.B.Singer et al.				X						
B. ubatubana (Hoehne) R.B.Singer et al.		X		X	X		X	X		X

No primeiro tratamento taxonômico para orquídeas do Brasil, Cogniaux (1904) reconheceu 11 espécies e cinco variedades morfologicamente relacionadas ao gênero *Maxillaria* (atualmente *Brasiliorchis*). Hoehne (1953) reconheceu 17 espécies e 19 variedades, incluindo três espécies e 14 variedades novas descritas. Em sua listagem de espécies de orquídeas brasileiras, Pabst e Dungs (1977) reconheceram 14 espécies divididas em três alianças: "*picta*", "*marginata*" e "*gracilis*", de acordo com caracteres vegetativos. Já Butzin e Senghas (1996) reconheceram apenas duas alianças, juntando as alianças "*picta*" e "*gracilis*" de Pabst e Dungs (1977) e mantendo "*marginata*".

Das espécies atualmente reconhecidas do gênero *Brasiliorchis*, cinco (*B. barbozae, B. gracilis, B. consanguinea, B. kautskyi e B. schunkeana*) são tradicionalmente mais fáceis de distinguir das outras espécies do gênero de acordo com caracteres morfológicos diagnósticos. Por outro lado, as dez espécies restantes (*B. chrysantha, B. heismanniana, B. marginata, B. monantha, B. moutinhoi, B. phoenicanthera, B. picta, B. polyantha, B. porphyrostele e B. ubatubana* - agrupamento *'Brasiliorchis picta'*) são morfologicamente mais similares, apresentando caracteres morfológicos aparentemente contínuos. No estudo morfométrico considerando caracteres vegetativos e florais, de seis espécies do gênero, Pinheiro e Barros (2009) não conseguiram identificar clusters correspondentes às espécies *B. chrysantha*, *B. marginata*, *B. picta, B. porphyrostele* e *B. ubatubana* - as outras espécies do agrupamento *'Brasiliorchis picta'* não foram amostradas. A única espécie claramente distinta foi *B. gracilis*, a qual não está incluída no agrupamento *'Brasiliorchis picta'*.

Posteriormente, Novello (2013) realizou um estudo de filogenia molecular para o gênero baseado em sequências nucleares e plastidiais. Os resultados indicam *B. schunkeana* como irmã de todas as outras espécies do gênero. *Brasiliorchis barbozae* foi reconhecida como um grupo monofilético distinto, corroborando com os caracteres morfológicos diagnósticos desta espécie, e possivelmente irmão das outras espécies de *Brasiliorchis*. A espécie *B. gracilis* não foi reconhecida como um grupo monofilético, não corroborando o estudo de morfometria de Pinheiro e Barros (2009). Contudo, nas análises concatenadas, alguns indivíduos de *B. gracilis* apresentaram-se como irmãos das espécies do complexo *B. picta*, sugerindo que a falta de resolução dos marcadores moleculares. As espécies morfologicamente mais homogêneas pertencentes ao complexo *B. picta* (*B. chrysantha*, *B. marginata*, *B. phoenicanthera*, *B. picta*, *B. porphyrostele* e *B. ubatubana*) não formaram clados monofiléticos distintos. *B. kautskyi* e *B. consanguinea*, que são morfologicamente mais diferenciadas dentro do complexo *B. picta*, apresentaram-se como clados inseridos no complexo *B. picta* (Novello, 2013).

A heterogeneidade do bioma da Mata Atlântica também representa um desafio adicional para o estudo de grupos ricos em espécies, como Orchidaceae. A variação dos caracteres vegetativos entre os hábitats leva a morfologias sobrepostas e complica a identificação das espécies tradicionalmente reconhecidas. Assim, estudos relacionados à biologia reprodutiva para estas espécies podem contribuir para a abordagem da biossistemática e sua relação com a taxonomia do grupo (Borba e Semir, 1999; Bronstein et al., 2014). Outro fator crucial nos estudos reprodutivos é considerar a fenologia das espécies. O conhecimento e a compreensão dos padrões fenológicos das espécies nos ecossistemas naturais são de interesse básico nos estudos ecológicos sobre a biodiversidade, produtividade e organização das comunidades e sobre as interações das plantas com a fauna, sendo também de grande importância em programas de conservação de recursos genéticos, manejo florestal e planificação de áreas silvestres (Morellato, 1995; Camacho e Orozco, 1998). Os estudos sobre fenologia reprodutiva de espécies de áreas florestais são necessários para fornecer parâmetros com vistas à conservação e exploração racional, conciliando sustentabilidade com economicidade (Fantini et al., 1992; Reis et al., 2000).

Especificamente, para a subtribo Maxillariinae, que contém cerca de 600 espécies neotropicais, o conhecimento sobre sistemas reprodutivos, fenologia reprodutiva, bem como sobre padrões de plasticidade em COVs florais foi pouco explorado (Kaiser, 1993; Singer, 2003; Flach et al., 2004). Portanto, este modelo de estudo é interessante, uma vez que a abordagem dos COVs florais e o sistema reprodutivo dentro do gênero poderia elucidar e contribuir à taxonomia (i.e. se as espécies são aparentadas e difíceis de identificar ou se são espécies-irmãs ou formam complexos de espécies), ecologia e bioconservação destas espécies. Dentre as subtribos de Orchidaceae com estudos referentes aos COVs florais, Maxillariinae apresenta poucos relatos disponíveis sobre plasticidade intra- e interespecífica de COVs florais (Kaiser, 1993; Flach et al., 2004). Desta forma, a determinação da composição química e o padrão de diversificação dos COVs, em espécies de *Brasiliorchis*, pode resultar em caracteres informativos para identificar linhagens reprodutivamente isoladas.

Kaiser (1993) foi o primeiro autor a descrever perfis químicos de COVs florais, considerando *Brasiliorchis* (*B. picta*), em que o principal COV encontrado foi o Estragol (42,5%). Posteriormente, Flach et al. (2004) identificaram os COVs de cinco espécies de *Brasiliorchis* (*B. chrysantha*, *B. gracilis*, *B. marginata*, *B. picta* e *B. ubatubana*) e encontraram alta diversidade interespecífica. Os compostos químicos mais representativos foram os grupos hidrocarbonetos, terpenóides, álcoois, aldeídos, cetonas e ésteres. Ao contrário de Kaiser

(1993), Flach et al. (2004) não encontraram Estragol na triplicata analisada. A pesquisa realizada por Flach et al. (2004) contribuiu muito para o conhecimento sobre a variação de COVs em *Brasiliorchis*, embora, tenha amostrado apenas uma parte das espécies do gênero. Aliada aos estudos de plasticidade em COVs florais, a abordagem sobre a polinização em orquídeas *Brasiliorchis* relata que as espécies *B. picta* e *B. marginata* são polinizadas pelas abelhas sem ferrão do gênero *Trigona* (Meliponinae) e que são, provavelmente, autoincompatíveis, devido à elevada taxa de aborto de frutos oriundos de autopolinização (Singer e Cocucci, 1999; Singer, 2003). No entanto, não foi confirmada a ocorrência de um sistema de autoincompatibilidade ou depressão endogâmica em *Brasiliorchis*, sendo, portanto, uma lacuna de conhecimento aos estudos reprodutivos deste gênero.

Neste estudo, descrevemos os sistemas reprodutivos de três espécies de *Brasiliorchis* com ampla distribuição geográfica, e confirmamos a presença de um sistema de autoincompatibilidade para a espécie *B. picta* (Capítulo 1). Adicionalmente, descrevemos e comparamos a nível interespecífico, a composição química de COVs florais para oito espécies do gênero (Capítulo 2). Especificamente, nossos objetivos foram: (1) Determinar os sistemas reprodutivos em *B. phoenicanthera*, *B. picta* e *B. porphyrostele*; (2) Descrever o tipo de autoincompatibilidade que ocorre em *B. picta*; (3) Determinar os COVs das fragrâncias florais em *B. barbozae*, *B. gracilis*, *B. marginata*, *B. phoenicanthera*, *B. picta*, *B. porphyrostele*, *B. schunkeana* e *B. ubatubana*, a fim de elucidar a questão de plasticidade de COVs florais em *Brasiliorchis*, relacionando ao sistema de polinização por engodo, isolamento reprodutivo e especiação.

CHAPTER I

First report on gametophytic self-incompatibility in *Brasiliorchis* orchids (Cymbidieae, Maxillariinae)

(This chapter is formatted according to the journal Plant Species Biology)

ABSTRACT

Gametophytic self-incompatibility system (GSI) is widely distributed in Angiosperms, and restricted to species-rich clades within the Orchidaceae. Atlantic Forest biome represents an additional challenge for the study of species-rich groups, such as Orchidaceae. The variation of vegetative characters between habitats leads to overlapping morphologies and complicates the identification of traditionally recognized species. This model is interesting, since the approach of floral reproductive system within the genus could elucidate and contribute to the taxonomy (i.e. whether the species are related and difficult to identify or whether they are sister species or form species complexes), ecology and bioconservation. We present data about the reproductive system of three species of *Brasiliorchis* and anatomical studies of ovary development after self- and cross-pollination in *B. picta*. As consequence, we here describe for the first time a GSI for genus *Brasiliorchis* within the also species-rich diverse subtribe Maxillariinae. Spontaneous self-pollination and emasculation set no fruits for none of the sampled species. Fruit set from cross-pollinations varied from 33.4-77.5%. A single fruit from self-pollination of *B. porphyrostele* was developed to completion. All the other fruits aborted between 14-21 days after pollination. Pollen tubes of aborted self-pollinated fruits never reach the ovules. Pollen tube growth patterns in *Brasiliorchis* are similar to subtribes Dendrobiinae and Pleurothallidinae. Studies of the self-incompatibility systems are needed to evaluate the role of their mechanisms in species diversification and evolution of reproductive strategies in Maxillariinae.

KEYWORDS

Brasiliorchis, fruit set, mating systems, *Maxillaria*, ovule development

INTRODUCTION

Self-Incompatibility (SI) is rare within family Orchidaceae, with 10% of species estimated as self-incompatible (Niu et al., 2017). Within the Epidendroideae, Maxillariinae is one of the most diverse orchid subtribes of the neotropics, with approximately 600 recognized species (Govaerts et al., 2005; Whitten et al., 2007). Despite high species diversity in Maxillariinae, there are only two species, *Lycaste cruenta* (Lindl.) Lindl. (syn. *Maxillaria skinneri* Lindl.) (East, 1940) and *Lycaste aromatica* (Graham) Lindl. (Hietz et al., 2006) which were suggested to have a self-incompatibility system. However, the authors did not develop an anatomical study to describe the type of self-incompatibility in these species.

Epidendroideae subfamily has 80% of the species of the family and the largest number of clades with described self-incompatible species. Self-incompatible species occur mainly in the Dendrobiinae, Oncidiinae and Pleurothallidinae subtribes (Table S1). Within Maxillariinae subtribe, one of the most diverse in the Neotropical region, with approximately 600 recognized species (Govaerts et al., 2005; Whitten et al., 2007), studies about the reproductive biology of these species (Singer, 2003) are still incipient, despite the high diversity of reproductive strategies (e.g., pseudomating, imitation of nesting and shelter sites, exploration of pollinator behavior) (Jersáková et al., 2006). Singer and Cocucci (1999) reported high fruit abortion in *Brasiliorchis picta* (Hook.) R.B.Singer, S.Koehler & Carnevali, but a detailed study on the reproductive system of this species is lacking. Except for *Brasiliorchis chrysantha* (Barb.Rodr.) R.B.Singer, S.Koehler & Carnevali, other species have been described as self-incompatible (Singer et al., 2007), but breeding system studies are still lacking.

SI are important pre- and post-zygotic reproductive barriers are known to occur at least in 40% of angiosperms (Rea & Nasrallah, 2008; Fujii et al., 2016). SI is expressed by the *S*-locus with multiple *S*-haplotypes (Takayama & Isogai, 2005; Iwano & Takayama, 2012). Each *S*-haplotype consists of, at least, two transcriptional units, known as male and female specificity

determinants: *S*-determinants. The discrimination of self-/nonself-recognition occurs through molecular interactions between these *S*-determinants (Takayama & Isogai, 2005; Iwano & Takayama, 2012). Three distinct forms of SI (GSI, SSI and LSI) are recognized, based on gynoecium response to pollen genotype (De Nettancourt, 1977; Richards, 1997; Gibbs, 2014; Oliveira & Maruyama, 2014). All the three types of SI have been described for the Orchidaceae, with most studies developed for subfamily Epidendroideae (Niu et al., 2018) (Tab. S1).

In Gametophytic Self-Incompatibility (GSI), the *S*-gene is expressed in the ovary and pollen tubes, so that the presence of equal alleles leads to an incompatibility reaction. GSI is widely distributed in angiosperms, associated with plants with highly secretory stigma (wet), in which SI reaction occurs along the style, causing degradation or early rupture of pollen tubes, in addition to irregular calluses depositions (Richards, 1997). In sporophytic self-incompatibility (SSI) there is dominance between *S*-haplotypes. Thus, incompatibility reactions are determined by the sporophyte (female) genotype. The incompatibility reaction avoids pollen grain germination or growth of pollen tubes. Incompatible pollen tubes rarely grow in the style and the self-incompatibility reaction occurs on the stigmatic surface (Oliveira & Gibbs, 1994). Plants with SSI usually present non-secretory stigma (dry), and are mainly restricted to some angiosperm families, such as Asteraceae, Brassicaceae, Betulaceae, Caryophyllaceae, Convolvulaceae and Polemoniaceae (Allen & Hiscock, 2008). Finally, late-acting self-incompatibility (LSI) acts in the ovary, apparently without affecting the growth of pollen tubes in the style (de Nettancourt, 1977, 1984; Seavey & Bawa, 1986). LSI can be expressed by the inhibition of incompatible pollen tubes in the ovary, before reaching the ovules; incompatible pollen tubes after they enter ovules; or the development of the zygote. LSI incompatibility reaction may also occur in tissues of the ovary, but the cause of self-incompatibility is still unknown (Seavey & Bawa, 1986; Bittencourt et al., 2003). LSI is particularly common in plants with small flowers and low-cost selection mechanisms (Seavey & Bawa, 1986; Oliveira &

Maruyama, 2014). GSI and SSI, however, are common in large and high-cost flowers, in which self-incompatibility is considered to increase cross-pollination and reduce inbreeding (Oliveira, 1998).

Brasiliorchis is one of the most taxonomically complex genera of Brazilian orchids. The absence of clear diagnostic morphological characters makes it difficult the development of studies in other areas of biological knowledge, in addition to being an obstacle to the commercialization of its species, which are of great ornamental value, as well as to the elaboration of measures for bioconservation and combating biopiracy (illegal trade) (Flores-Palacios and Valencia-Díaz, 2007; Swarts and Dixon, 2009). Of the currently recognized species of the genus *Brasiliorchis*, five (*B. barbozae*, *B. gracilis*, *B. consanguinea*, *B. kautskyi* and *B. schunkeana*) are traditionally easier to distinguish from other species of the genus according to diagnostic morphological characters. On the other hand, the ten remaining species (*B. chrysantha*, *B. heismanniana*, *B. marginata*, *B. monantha*, *B. moutinhoi*, *B. phoenicanthera*, *B. picta*, *B. polyantha*, *B. porphyrostele* and *B. ubatubana* - grouping *'Brasiliorchis picta'*) are morphologically more similar, presenting apparently continuous morphological characters. In the morphometric study considering vegetative and floral characters of six species of the genus, Pinheiro and Barros (2009) were unable to identify clusters corresponding to the species *B. chrysantha*, *B. marginata*, *B. picta*, *B. porphyrostele* and *B. ubatubana* - others species of the *'Brasiliorchis picta'* grouping were not sampled. Only clearly distinct species was *B. gracilis*, which is not included in the *'Brasiliorchis picta'*.

Subsequently, Novello (2013) carried out a molecular phylogeny study for the genus based on nuclear and plastid sequences. The results indicate *B. schunkeana* as sister to all other species of the genus. *Brasiliorchis barbozae* was recognized as a distinct monophyletic group, corroborating the diagnostic morphological characters of this species, and possibly a brother to the other *Brasiliorchis* species. The species *B. gracilis* was not recognized as a monophyletic

group, not corroborating the morphometric study by Pinheiro and Barros (2009). The morphologically more homogeneous species belonging to the *B. picta* complex (*B. chrysantha*, *B. marginata*, *B. phoenicanthera*, *B. picta*, *B. porphyrostele* and *B. ubatubana*) did not form distinct monophyletic clades. *B. kautskyi* and *B. consanguinea*, which are morphologically more differentiated within the *B. picta* complex, were presented as clades within the *B. picta* complex (Novello, 2013).

The heterogeneity of the Atlantic Forest biome also represents an additional challenge for the study of species-rich groups such as Orchidaceae. The variation of vegetative characters between habitats leads to overlapping morphologies and complicates the identification of traditionally recognized species. Thus, studies related to reproductive biology for these species can contribute to the approach of biosystematics and its relationship with the taxonomy of the group (Borba and Semir, 1999; Bronstein et al., 2014). Therefore, this model is interesting, since the approach of the reproductive system within the genus could elucidate and contribute to the taxonomy (i.e. whether the species are related and difficult to identify or whether they are sister species or form complexes of species), ecology and bioconservation of these species. Furthermore, many species of Atlantic Forest are key-species in the biome, contributing to the survival of other species. Another point is the reduction or loss of the gene flow between different species, interfering in the action of reproductive barriers and decrease in the biological diversity. Such topics were selected for the present study, aiming at the importance of these barriers in endemic species found in biomes highly modified by anthropic activities.

In this study, our goals were: (1) to describe data on the reproductive system of the genus *Brasiliorchis*; *Brasiliorchis phoenicanthera* (Barb.Rodr.) R.B.Singer, S.Koehler & Carnevali, *B. picta* and *Brasiliorchis porphyrostele* (Rchb.f.) R.B.Singer, S.Koehler & Carnevali). Furthermore, as consequence of the results obtained from reproductive biology quantitative

data (2) we describe for the first time the occurrence of a gametophytic self-incompatibility mechanism for the genus *Brasiliorchis*, in *B. picta*.

MATERIAL AND METHODS

Studied species

Species of *Brasiliorchis* occur mainly in South American Atlantic Forest and surrounding vegetation, mainly in Brazil, from the States of Rio Grande do Sul to Bahia and Pernambuco extending to Misiones, Argentina (Hoehne, 1953; Pabst & Dungs, 1977; Waechter, 1996; Toscano de Brito & Cribb, 2005; Singer et al., 2007; Barros et al., 2015). Species have a sympatric distribution, sharing the same pollinator (genus *Trigona*, stingless bee) (Singer & Cocucci, 1999; Singer, 2003). There are no hybridization studies for the genus to date. *Brasiliorchis* can be easily identified by their bifoliate, strap-like leaves and ridged to sulcate pseudobulbs, commonly with reddish or brown roots. Flowers are campanulate, showy and long-lasting anthesis, sweety-scented like honey, commonly yellowish or greenish in color and rewardless (Singer et al., 2007) (Figs. 1A-F).

Brasiliorchis picta is the most widespread species of *Brasiliorchis*, with abundant specimens in cultivation in orchid nurseries of Brazil. Its flowers are externally yellow with vinaceous spots grouped in irregular areas. Petals are narrow and acuminated, usually crossing the column. The lip is white, with many vinaceous spots at the base and sparse on the apex. *B. phoenicanthera* has light yellow petals and sepals and a creamy or white lip. Its flowers are smaller than *B. picta*, with no vinaceous spots on the petals and sepals. *Brasiliorchis porphyrostele* has flowers externally yellowish, and internally greenish-yellow. Sepals are oblong and slightly curved. The lip is creamed-colored and with many vinaceous lines. Column is also vinaceous (Hoehne, 1953; Singer et al., 2007). Vouchers were deposited at herbarium

UEC (State University of Campinas, UNICAMP, Brazil) and plants are in cultivation at the orchid nursery of Instituto de Botânica (São Paulo, Brazil) (Tab. S2).

Reproductive system

To evaluate the dependence on pollinators for reproduction, we performed treatments for pollination description in the nursery orchid collection of the Núcleo de Pesquisa Orquidário do Estado Dr. Frederico Carlos Hoehne (Instituto de Botânica, São Paulo, Brazil). We performed artificial treatments of self-pollination (SP, pollinia from the same flower), cross-pollination (CP) and emasculation (removal of pollinia to verify asexual reproduction). We also controlled for spontaneous self-pollination observing unmanipulated flowers. Flowers were bagged before anthesis in spontaneous self-fertilization/control and emasculation treatments. We used a whole pollinarium in each experimental pollination event. All cross-pollination treatments considered specimens of different localities in Brazil (Tab. S2). Treatments were performed in *N*= 30 specimens for *B. phoenicanthera*, *N*= 31 for *B. picta* and *N*= 29 for *B. porphyrostele*. In each specimen/individual, we selected four flowers, being one to each treatment. The order of treatments applied to each inflorescence was random and we used first to third day old flowers. We used the number of mature fruits to estimate reproductive success and recorded the fruit set when fruits were dehiscent. We also registered the initial and final month of flowering specimens and the period for fruit development for two consecutive years. To evaluate the degree of self-incompatibility of the three species sampled, the index of self-incompatibility (ISI) was calculated using the formula ISI = extent of fruit set in self-pollinated flowers/extent of fruit set in cross-pollinated flowers. Thus, a plant is considered fully self-compatible (FSC) when ISI is ≥1, partially self-incompatible when ISI is ≥0.2 but <1, mostly self-incompatible when ISI is <0.2 but >0 and fully self-incompatible (SI) when ISI is 0 (Zapata & Arroyo, 1978; Androulakis & Loupassaki, 1990) (Tab. 1).

Anatomical studies of self-incompatibility in *B. picta*

To identify and characterize a putative self-incompatibility mechanism in *Brasiliorchis*, we performed anatomical studies of ovary development after self- and cross-pollination treatments in *B. picta*. We performed artificial self- and cross-pollinations on the first day of anthesis. For cross-pollinations, we used pollinia from flowers of plants from different populations. Self-pollinated flowers were collected every 24 h (*N*= 15) and cross-pollinated flowers every 72 h (*N*= 13). We use one flower for each collection day. These intervals were defined according to personal observations on fruit abortion period.

Flowers were fixed in FAA (37% formaldehyde, glacial acetic acid and 70% ethanol, 90:5:5 v/v). We dehydrated the samples using an ethanol series and then infiltrated and polymerized with hydroxyethyl methacrylate (Historesin®, Leica, Germany) according to the supplier's instructions. We prepared longitudinal 5 μm thick sections using disposable blades and a Leica rotary microtome. We assembled and stained the sections with aniline blue (0.1%) in water soluble for pollen tube development (Martin, 1959) and toluidine blue for ovary observations (Sakai, 1973). We recorded the pollen tubes development in a digital camera Olympus DP71 coupled to microscopy Olympus BX51.

RESULTS

We observed flowering overlapping among all three species: *B. phoenicanthera* (June - September), *B. picta* (April - July), and *B. porphyrostele* (May - July). Anthesis in *Brasiliorchis* lasts from 10 to 15 days. Fruit ripening occurred 79 - 140 days after pollination (DAP) for *B. phoenicanthera*, 91 - 134 DAP for *B. picta*, and 94 - 142 DAP for *B. porphyrostele* (Tab. 1). Spontaneous self-pollination and emasculation treatments set no fruits for none of the sampled species. A single fruit from SP treatment of *B. porphyrostele* was developed to completion, and fruit set from CP varied from three species (33.4 – 77.5%) (Tab. 1). ISI for *B. phoenicanthera*

and *B. picta* = 0 (fully self-incompatible), while *B. porphyrostele* = 0.08 (mostly self-incompatible) (Tab. 1).

TABLE 1. Number of fruits developed after self- and cross-pollination treatments and Index of Self-Incompatibility (ISI) in three sampled species of *Brasiliorchis* R.B.Singer, S.Koehler & Carnevali. Percentage of the fruit set is indicated in parentheses.

Species	Self-pollination	Cross-pollination	ISI
B. phoenicanthera	0/30 (0)	10/30 (33.33)	0
B. picta	0/31 (0)	24/31 (77.42)	0
B. porphyrostele	1/29 (3.45)	12/29 (41.38)	0.08

We observed a developed placentae and ovule primordia initiating development after 1 DAP (Figs. 2A-B). At 3 DAP, the placentae of SP and CP flowers expand into the ovary locules causing an increase in fruit volume (Figs. 2C-D). Pollen grain germination on stigma already initiated in CP flowers at this time, but not in SP ones (Figs. 2E-F). At 5-6 DAP, CP fruit placentae have ovule primordia with conspicuous nuclei and intense cell division, while in SP fruits (Fig. 2G) their cells have less dense cytoplasm (Fig. 2H). In both treatments at this time, pollen tubes contact stigmatic secretory cells which detach throughout the stigma and stylar canal (Figs. 2I-J). At 5-6 DAP, pollen tubes grow through secretory cells of the stylar canal, although further ovule development was not observed at this time in both CP and SP flowers (Figs. 3A-B). At 9-10 DAP, the placental region is fully developed in both treatments (Figs. 3C-D) and cell nuclei from CP ovule primordia remain conspicuous (Fig. 3E). Ovules from SP fruit are more vacuolized but with inconspicuous nuclei (Fig. 3F). Pollen tubes grow vigorously through the stylar canal after 12-13 DAP in CP fruits, while in SP fruits they show irregular patterns and dense cytoplasm at the median portion of the stylar canal and cease growth (Figs. 3G-J). Ovules at 12-13 DAP in CP fruits continue to differentiate (Figs. 4A, C) while ovule primordia and cells in the placental region degenerate in SP (Figs. 4B, D). At 15-16 DAP, CP fruits increase in diameter and the placenta occupies most of the locule area (Fig. 4E). SP fruits

remain attached to the floral stalk at this time, but placenta cells cease to further divide (Fig. 4F). At this time, a vast amount of pollen tubes still grows through the stylar canal in CP fruits (Fig. 4G), while pollen tubes in SP fruits deteriorate and never reach the ovary (Fig. 4H). At 21 DAP, pollen tubes of CP fruits reach the first ovule primordia (Fig. 4I), while pollen tubes of SP fruits never reach the ovule (Fig. 4J) and abort between 14-21 DAP. In ovule primordia of CP fruits, terminal subdermal cells differentiate into archesporial cells with conspicuous nuclei (Figs. 5A-B). At this time, we also observe cells with dense cytoplasm in CP fruits (Fig. 5A). At 56 DAP, we observe developed seeds with embryos and a clear differentiated suspensor (Figs. 5C-D). At 64 DAP, seeds have well developed embryos with a degraded suspensor and are protected by a delicate integument composed by cells impregnated with lignin (Figs. 5E-F).

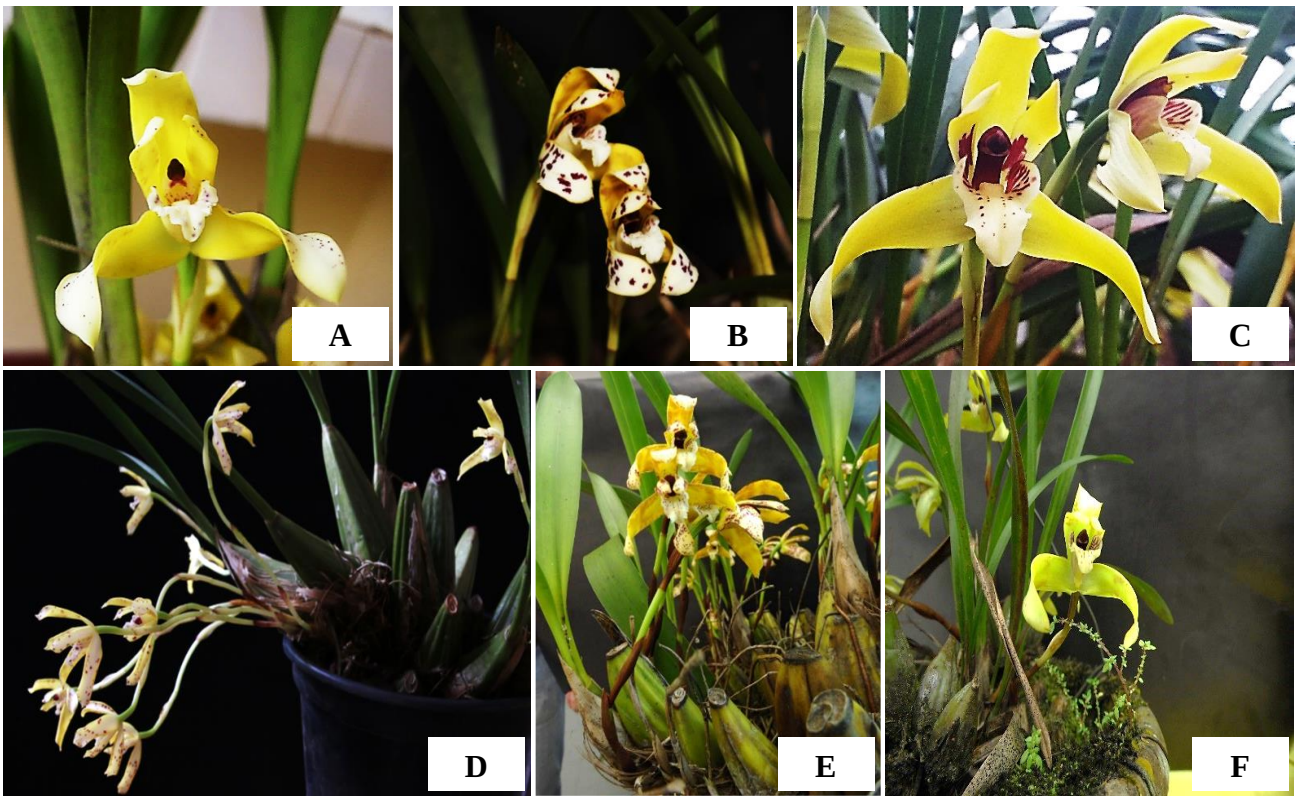

FIGURE 1. *Brasiliorchis* species used for reproductive and anatomical studies: A-C represent the flowers (reproductive structure) of *Brasiliorchis phoenicanthera* (Barb.Rodr.) R.B.Singer, S.Koehler & Carnevali, *Brasiliorchis picta* (Hook.) R.B.Singer, S.Koehler & Carnevali and *Brasiliorchis porphyrostele* (Rchb.f.) R.B.Singer, S.Koehler & Carnevali, respectively. D-F represent the leaves and pseudobulbs (vegetative structures) of *B. phoenicanthera*, *B. picta* and *B. porphyrostele*, respectively. A and D: L.F.Varella; B-C and F: own authorship; E: S.Koehler.

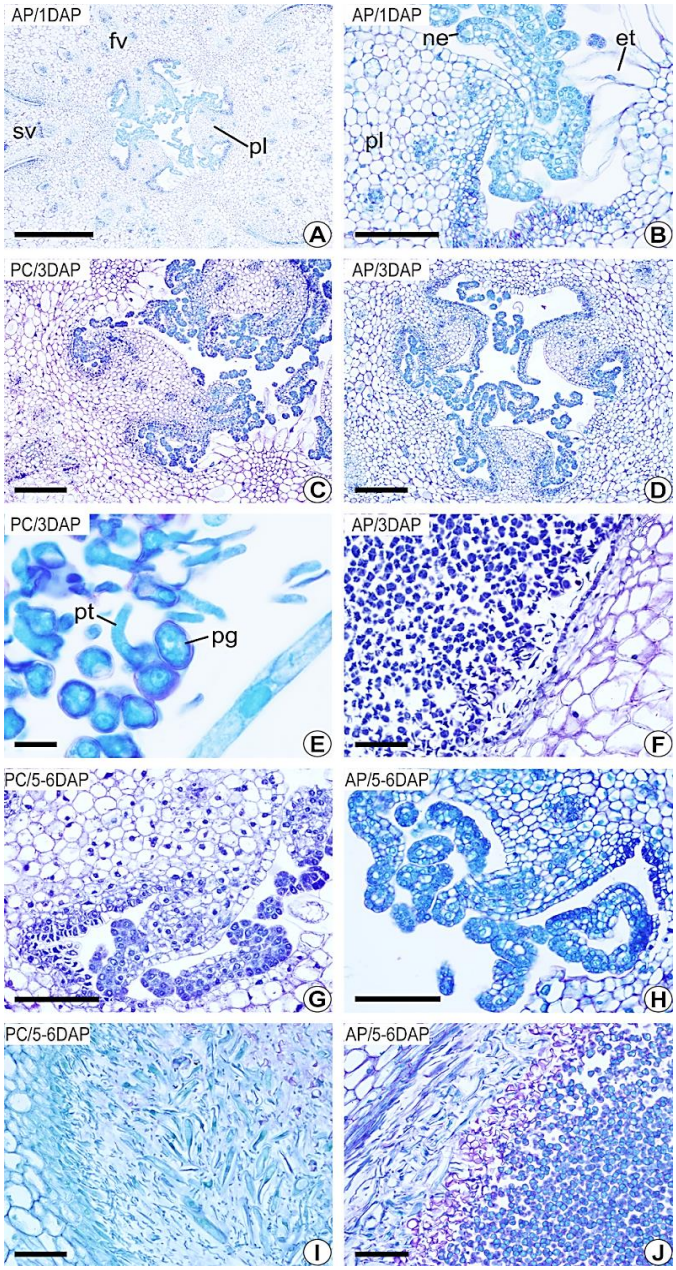

FIGURE 2. Light microscopy of transversal sections of ovary on different days after controlled pollination treatments (DAP) in *Brasiliorchis picta* (Hook.) R.B.Singer, S. Koehler & Carnevali. Cross-Pollination (PC) and Self-Pollination (AP). A-B. Developed placentae and ovule primordia initiating development after 1 DAP. Note the placentae with fertile and sterile valves (A) and nucellar epiderms and elaters (B). C-D. Placentae of AP and PC flowers expand into the ovary locules at 3 DAP. E-F. Pollen grain germination on stigma already initiated in PC flowers at this time, but not in AP ones, after 3 DAP. G. Fruit placentae with ovule primordia with conspicuous nuclei and intense cell division (5-6 DAP). H. AP fruits have cells with less dense cytoplasm (5-6 DAP). I-J. In both treatments, pollen tubes contact stigmatic secretory cells which detach throughout the stigma and stylar canal (5-6 DAP). et = elaters; fv = fertile valve; ne = nucellar epidermis; pg= pollen grain; pl = placenta; pt = pollen tube; sv = sterile valve. Bars: (A) 500 μm; (B) 100 μm; (C-D) 200 μm; (E) 20 μm; (F-J) 100 μm.

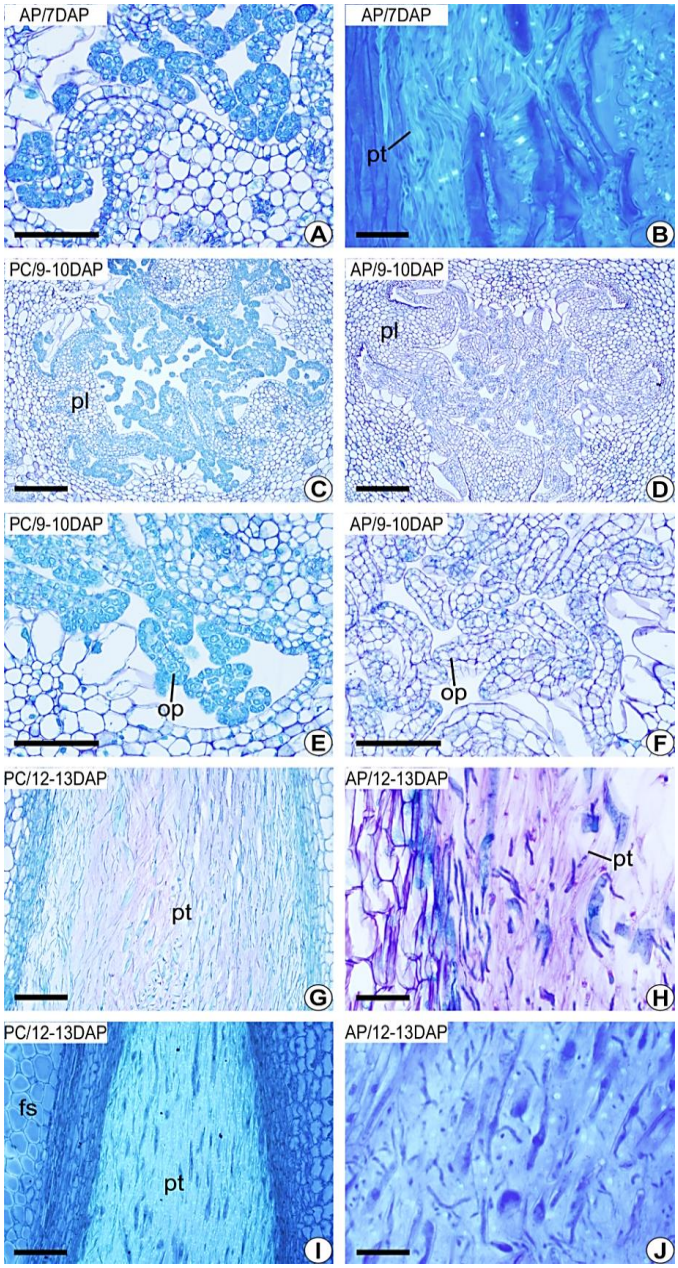

FIGURE 3. Light microscopy of transversal sections of ovary (A, C-F), light microscopy of longitudinal sections of stylar canal (G-H, J) and longitudinal sections of stylar canal using fluorescence microscopy in UV light, where pollen tubes are evidenced (B, I) on different days after controlled pollination treatments (DAP) in *Brasiliorchis picta* (Hook.) R.B.Singer, S. Koehler & Carnevali. Cross-Pollination (PC) and Self-Pollination (AP). A-B. At 5-6 DAP, pollen tubes grow through secretory cells of the stylar canal, although further ovule development was not observed at this time in both PC and AP flowers. C-D. At 9-10 DAP, the placental region is fully developed in both treatments. E. Cell nuclei from PC ovule primordia remain conspicuous. F. Ovules from AP fruit are more vacuolized but with inconspicuous nuclei. G-J. Pollen tubes grow vigorously through the stylar canal after 12-13 DAP in PC fruits, while in AP fruits they show irregular patterns and dense cytoplasm at the median portion of the stylar canal and cease growth. fs = stylus; op = ovule primordium; pl = placenta; pt = pollen tube. Bars: (A, E, G) 100 µm; (B, H, J) 50 µm; (C-D, I) 200 µm.

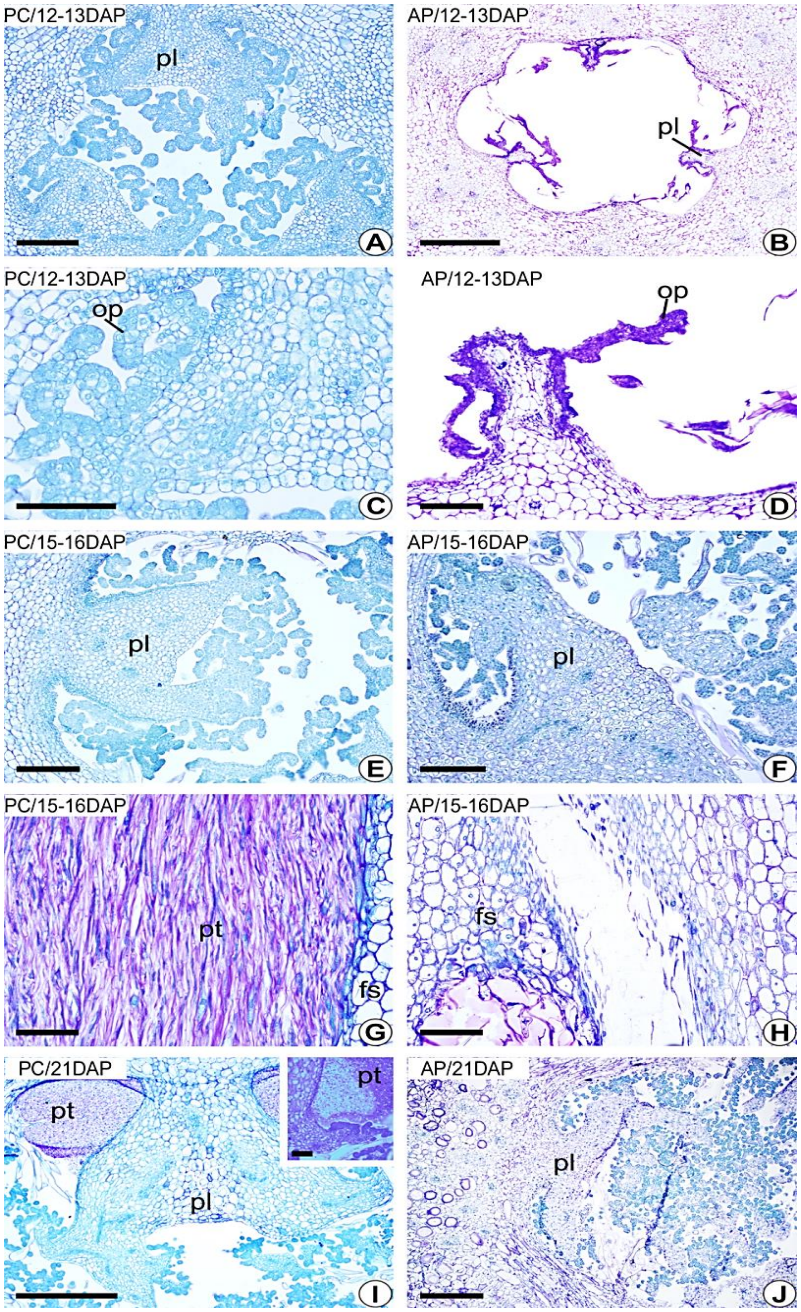

FIGURE 4. Light microscopy of transversal sections (A-F, I-J) and longitudinal sections (G-H) of ovary and stylar canal, respectively, on different days after controlled pollination treatments (DAP) in *Brasiliorchis picta* (Hook.) R.B.Singer, S. Koehler & Carnevali. Cross-Pollination (PC) and Self-Pollination (AP). A, C. Ovules at 12-13 DAP in PC fruits continue to differentiate. B, D. Ovule primordia and cells in the placental region degenerate in AP. E. At 15-16 DAP, PC fruits increase in diameter and the placenta occupies most of the locule area. F. AP fruits remain attached to the floral stalk at this time, but placenta cells cease to further divide. G. At this time, a vast amount of pollen tubes still grows through the stylar canal in PC fruits. H. Pollen tubes in AP fruits deteriorate and never reach the ovary. I. At 21 DAP, pollen tubes of PC fruits reach the first ovule primordia. J. Pollen tubes of AP fruits never reach the ovule and abort between 14-21 DAP. Detail in I. Fluorescence microscopy in UV light, where the pollen tubes are evidenced. fs = stylus; op = ovule primordium; pl = placenta; pt = pollen tube. Bars: (A, E, H) 200 μm; (B, F, I-J) 500 μm; (C-D, G, I-detail) 100 μm.

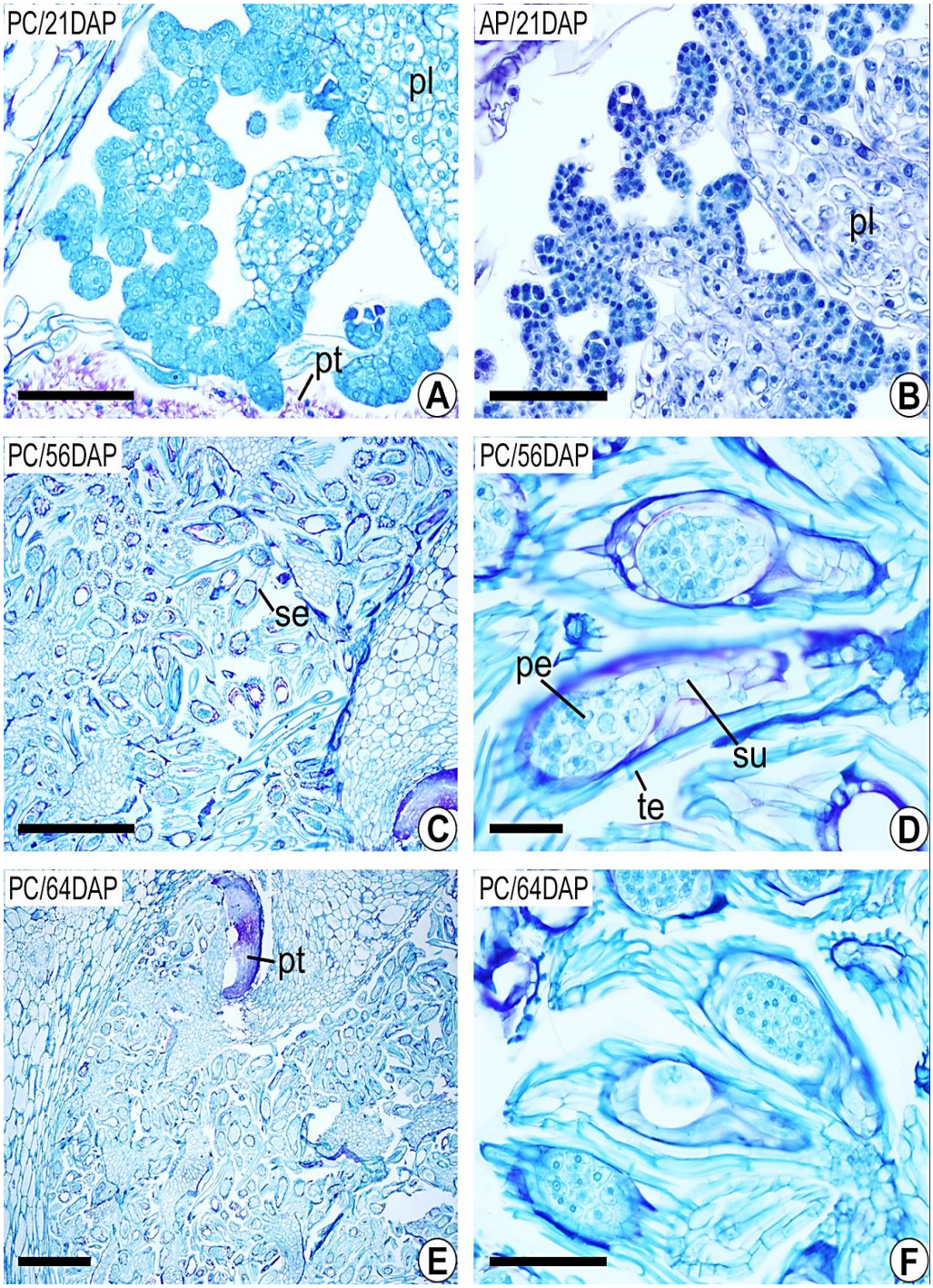

FIGURE 5. Light microscopy of transversal sections (A-F) of ovary on different days after controlled pollination treatments (DAP) in *Brasiliorchis picta* (Hook.) R.B.Singer, S. Koehler & Carnevali. Cross-Pollination (PC) and Self-Pollination (AP). A-B. In ovule primordia of PC fruits, terminal subdermal cells differentiate into archesporial cells with conspicuous nuclei. A. At this time, we also observe cells with dense cytoplasm in PC fruits. B. Abortion in AP fruits. C-D. At 56 DAP, we observe developed seeds with embryos and a clear differentiated suspensor. E-F. At 64 DAP, seeds have well developed embryos with a degraded suspensor and are protected by a delicate integument composed by cells impregnated with lignin. pe = properly embryo; pl = placenta; pt = pollen tube; se = seed; su = suspensor; te = integument. Bars: (A-B, F) 100 μm; (C, E) 500 μm; (D) 50 μm.

DISCUSSION

We report for the first time Gametophytic Self-Incompatibility for subtribe Maxillariinae and describe its reproductive system plus two additional species, *B. phoenicanthera* and *B. porphyrostele*. Our results suggested a single fruit from SP treatment of *B. porphyrostele* was developed to completion, indicating that the self-incompatibility mechanism in *Brasiliorchis* can act in different levels - from total self-incompatibility (*B. phoenicanthera* and *B. picta*) to strong self-incompatibility (*B. porphyrostele*). Fruit set from CP varied from three species (33.4 – 77.5%) (Tab. 1). Furthermore, our results suggested the occurrence of a strong self-incompatibility system in all three studied species, as fruits aborted 14 - 21 days after self-pollination and the ovules did not differentiate. The rapid abortion in *Brasiliorchis* species, initially, made us suggest that sporophytic self-incompatibility would be occurring within the genus. In this case, the incompatibility reaction would be manifested in the floral stigma with the absence of pollen grains germination. However, it would only be possible to affirm this hypothesis from plant anatomy experiments. Anatomical studies for *B. picta* showed that pollen tubes from self-pollinated flowers stopped growing in the stylar canal and pollen tubes never reach the ovary. Also, there was irregular morphology of pollen tubes in the stylar canal despite regular swollen reaction and closure of the stigma and pollen tube germination. The absence of fruits in flowers tested for spontaneous self-pollination and emasculation indicates that none of the species is able to develop fruits by autonomous self-pollination or asexual reproduction mechanisms, respectively. Thus, pollination is necessary for the fruit set in these species.

Different systems of self-incompatibility (SSI, GSI and LSI) are suggested to occur in at least 15 subtribes, yet it has been confirmed only for Dendrobiinae, Pleurothallidinae, Oncidiinae (*Rodriguezia granadensis* (Lindl.) Rchb.f.) and one species within subfamily Cypripedioideae (*Cypripedium calceolus* L.) (Tab. S1). Available studies for Pleurothallidinae

and *Dendrobium* (Dendrobiinae) species point to variable strengths of GSI (weak/partial or strong/strict) (Borba et al., 2001; Niu et al., 2018; Duarte et al., 2020). In *Masdevallia infracta* Lindl., *Octomeria*, *Stelis*, *Specklinia*, and *Anathallis*, pollen after pollination does not germinate, being, then, considered SSI species (Borba et al., 2011), while self-pollination of 26 species of *Restrepia* revealed that pollen tubes grow only to into one-third of the ovary (Miller et al., 2015). Morphological patterns of pollen tube growth in *Acianthera adamantinensis* (Brade) F.Barros and *Acianthera fabiobarrosii* (Borba & Semir) Borba and *Dendrobium longicornu* Lindl. and *Dendrobium chrysanthum* Wall. ex Lindl. are similar to *B. picta*, as pollen tube growth ceases in the style (Borba et al., 2001; Niu et al., 2018). In *Restrepia*, less pollen tubes grow in the stylar canal in SP flowers when compared with CP ones, but fruits with empty seeds are formed as a small number of pollen tubes may penetrate the apical region of the ovary (Miller et al., 2015). Borba et al. (2001) also reports the occurrence of viable seeds in fruits of *Acianthera* and weak or partial GSI. It is possible that other clades within Maxillariinae have SI systems with various strengths, as fruit abortion after SP treatments have been observed for other genera (R.B.Singer and S.Koehler, pers.obs.).

SI species are expected to have lower rates of both speciation and extinction than self-compatible species (Takebayashi & Morrell, 2001). This is because, SI species have higher outcrossing rates and maintain larger effective population sizes, while self-compatible species generally have smaller effective population sizes, increased rates of fixation of slightly deleterious mutations and lowered rates of fixation of beneficial alleles (Charlesworth, 2003; Wright et al., 2008). A total of 104 taxa of orchids suggested self-incompatibility systems, and of these 97 were described as GSI (93.27%), five LSI (4.81%) and two SSI (1.92%) (Tab. S1). Of these, 102 taxa (98.07%) had their self-incompatible system confirmed by anatomical or morphological studies. Only the studies with *C. calceolus* (Pedersen et al., 2012) and *R. granadensis* (Ospina-Calderón et al., 2015) did not perform anatomical and/or morphological

analyzes to confirm SI system. SI is unknown to occur in subfamilies Apostasioideae and Vanilloideae and it is rare in Orchidoideae, as the occurrence of SI was only suggested in *Galearis spectabilis* (Dieringer, 1982). For Cypripedioideae, a single SI species is known, *C. calceolus* (LSI system, Pedersen et al., 2012). All described GSI species belong to high species-diverse subtribes of Epidendroideae: five species of *Pleurothallis* subgen. *Specklinia* sect. *Muscosae* (Epidendroideae, Pleurothallidinae) (Luer, 1986, 1999; Borba et al., 2001; Pridgeon & Chase, 2001; Borba, 2003), *Acianthera johannensis* (Barb.Rodr.) Pridgeon & M.W.Chase (Epidendroideae, Pleurothallidinae) (Borba et al., 2001), nine species of *Dendrobium* (Epidendroideae, Dendrobiinae) (Niu et al., 2018) and 21 species of *Restrepia* (Epidendroideae, Pleurothallidinae) (Millner et al., 2015). In Oncidiinae, Ospina-Calderón et al. (2015) suggested a GSI system for *R. granadensis* based on complete fruit abortion in flowers submitted to the self-pollination, being similar to *Brasiliorchis* species, however they did not perform anatomical and morphological studies. Singer and Koehler (2003) reported the occurrence of secretory cells of the stigma (Eleutherocytes) in *Notylia nemorosa* Barb.Rodr., being associated with the reaction of self-incompatibility, suggesting the occurrence of some type of SI in this species. However, further study is lacking for these species. *Dendrobium* is the best studied genus, with 66 species confirmed as self-incompatible (Tab. S1). Contrary to Pleurothallidinae species, all of them have a GSI system, except for *Dendrobium denneanum* Kerr and some populations of *D. longicornu* Lindl. which have LSI and SSI systems, respectively.

The adaptations found in Orchidaceae favoring cross-pollination, and plant-pollinator relationships are responsible for the enormous variety of species in Maxillariinae (Dodson, 1962). A myriad of orchid species is self-compatible, but self-fertilization commonly is avoided by preventing pre-pollination mechanisms (Tremblay et al., 2005). Studies that investigate self-incompatibility mechanisms are essential in understanding the action of reproductive barriers and speciation. Changes in compatibility levels intensely affect the levels of reproductive

isolation between populations, and the relationship between SI and SC may contribute to the reproductive barriers among distinct lineages, leading to speciation or extinction processes (Hiscock et al., 1998; Brandvain & Haig, 2005). Reproduction systems are affected by several external factors to the plants, such as vectors and pollination behavior, and, within plants, by phenological factors and allocation of resources within the fruits (Millar et al., 2000). Self-incompatibility also affects the plant reproduction system. These factors can generate variations in the outcrossing rate and paternity correlation in fruits of different specimens, and between specimens intra- and interpopulations. Knowledge of variation in the reproduction systems in populations of a species is of fundamental importance for the design of strategies for the conservation and genetic improvement (Ohashi et al., 1992), since it allows the application of more effective sampling methods and the use of more realistic mathematical models in the study of the quantitative inheritance of characters of economic interest (Sebbenn, 2000). This study brings new reproductive information about the action of self-incompatibility system to the genus *Brasiliorchis*. Further studies considering clades with no species delimitation studies are needed to test the hypothesis of self-incompatibility systems as drivers of speciation in the subtribe Maxillariinae.

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SUPPLEMENTARY MATERIAL

TABLE S1. Species of Orchidaceae described as self-incompatible (SSI: sporophytic self-incompatibility, GSI: gametophytic self-incompatibility, LSI: late-acting self-incompatibility). Species names follow the World Checklist of Selected Plant Families (Royal Botanical Gardens, Kew, UK). Names used in the original studies are indicated in parentheses.

Species	Subfamily	Subtribe	Self-incompatibility system	Reference
Cypripedium calceolus L.	**CYPRIPEDIOIDEAE**	Cypripediinae	LSI	Pedersen et al., 2012
Galearis spectabilis (L.) Raf.	**ORCHIDOIDEAE**	Orchidinae	undescribed	Dieringer, 1982
Acampe pachyglossa Rchb.f. (syn. *Acampe pachyglossa* subsp. *reschiana* (Rchb.f.) Senghas)	**EPIDENDROIDEAE**	Aeridinae	undescribed	Agnew, 1986
Acampe praemorsa (Roxb.) Blatt. & McCann		Aeridinae	undescribed	Agnew, 1986
Acianthera adamantinensis (Brade) F.Barros (syn. *Pleurothallis adamantinensis* Brade)		Pleurothallidinae	GSI (strong/strict)	Borba et al., 2001
Acianthera fabiobarrosii (Borba & Semir) Borba (syn. *Pleurothallis fabiobarrosii* Borba & Semir)		Pleurothallidinae	GSI (strong/strict)	Borba et al., 2001
Acianthera johannensis (Barb. Rodr.) Pridgeon & M.W.Chase (syn. *Pleurothallis johannensis* Barb. Rodr.)		Pleurothallidinae	GSI (weak/partial)	Borba et al., 2001
Acianthera johannensis (Barb. Rodr.) Pridgeon & M.W.Chase (syn. *Pleurothallis johannensis* Barb. Rodr.)		Pleurothallidinae	GSI (weak/partial)	Borba et al., 2001
Acianthera limae (Porto & Brade) Pridgeon & M.W.Chase		Pleurothallidinae	LSI or inbreeding	Melo et al., 2011
Acianthera modestissima (Rchb.f. & Warm.) Pridgeon & M.W.Chase		Pleurothallidinae	LSI or inbreeding	Melo et al., 2011
Acianthera ochreata (Lindl.) Pridgeon & M.W.Chase (syn. *Pleurothallis ochreata* Lindl.)		Pleurothallidinae	GSI (weak/partial)	Borba et al., 2001
Acianthera prolifera (Herb. ex Lindl.) Pridgeon & M.W.Chase (syn. *Acianthera hamosa* (Barb.Rodr.) Pridgeon & M.W.Chase)		Pleurothallidinae	LSI or inbreeding	Melo et al., 2011
Acianthera teres (Lindl.) Borba (syn. *Pleurothallis teres* Lindl.)		Pleurothallidinae	GSI (weak/partial)	Borba et al., 2001
Anathallis liparanges (Rchb.f.) Luer (syn. *Anathallis heterophylla* Barb.Rodr.)		Pleurothallidinae	undescribed	Gontijo et al., 2010

Species	Subfamily	Subtribe	Self-incompatibility system	Reference
Anathallis microphyta (Barb.Rodr.) C.O.Azevedo & van den Berg		Pleurothallidinae	undescribed	Gontijo et al., 2010
Anathallis rubens (Lindl.) Pridgeon & M.W.Chase		Pleurothallidinae	undescribed	Gontijo et al., 2010
Anathallis sclerophylla (Lindl.) Pridgeon & M.W.Chase		Pleurothallidinae	undescribed	Gontijo et al., 2010
Angraecum cultriforme Summerh.		Angraecinae	undescribed	Agnew, 1986
Cattleya warneri T.Moore ex R.Warner		Laeliinae	undescribed	Stort and Martin, 1980
Coelogyne fimbriata Lindl.		Coelogyninae	undescribed	Liu et al., 2013
Coelogyne rigida C.S.P.Parish & Rchb.f.		Coelogyninae	undescribed	Liu et al., 2013
Cyrtochilum cimiciferum (Rchb.f.) Dalström (syn. *Oncidium cimiciferum* (Rchb.f.) Beer)		Oncidiinae	undescribed	East, 1940; Tremblay et al., 2005; Castro and Singer, 2019
Dendrobium aciculare Lindl.		Dendrobiinae	GSI/ Eleutherocytes	Johansen, 1990
Dendrobium acinaciforme Roxb.		Dendrobiinae	GSI/ Eleutherocytes	Johansen, 1990
Dendrobium albosanguineum Lindl. & Paxton		Dendrobiinae	GSI/ Eleutherocytes	Johansen, 1990
Dendrobium aloifolium (Blume) Rchb.f.		Dendrobiinae	GSI/ Eleutherocytes	Johansen, 1990
Dendrobium alterum Seidenf.		Dendrobiinae	GSI/ Eleutherocytes	Johansen, 1990
Dendrobium aphyllum (Roxb.) C.E.C.Fisch		Dendrobiinae	GSI/ Eleutherocytes	Johansen, 1990
Dendrobium bicameratum Lindl.		Dendrobiinae	GSI/ Eleutherocytes	Johansen, 1990
Dendrobium bilobulatum Seidenf.		Dendrobiinae	GSI/ Eleutherocytes	Johansen, 1990
Dendrobium blumei Lindl.		Dendrobiinae	GSI/ Eleutherocytes	Johansen, 1990
Dendrobium brevimentum Seidenf.		Dendrobiinae	GSI/ Eleutherocytes	Johansen, 1990
Dendrobium cariniferum Rchb.f.		Dendrobiinae	GSI/ Eleutherocytes	Johansen, 1990
Dendrobium chrysotoxum Lindl.		Dendrobiinae	GSI/ Eleutherocytes	Johansen, 1990

Species	Subfamily	Subtribe	Self-incompatibility system	Reference
Dendrobium crystallinum Rchb.f.		Dendrobiinae	GSI/ Eleutherocytes	Johansen, 1990
Dendrobium compactum Rolfe ex Hemsl.		Dendrobiinae	GSI/ Eleutherocytes	Johansen, 1990
Dendrobium crumenatum Sw.		Dendrobiinae	GSI/ Eleutherocytes	Johansen, 1990
Dendrobium denudans D.Don		Dendrobiinae	GSI/ Eleutherocytes	Johansen, 1990
Dendrobium devonianum Paxton		Dendrobiinae	GSI/ Eleutherocytes	Johansen, 1990
Dendrobium distichum (C.Presl) Rchb.f.		Dendrobiinae	GSI/ Eleutherocytes	Johansen, 1990
Dendrobium draconis Rchb.f.		Dendrobiinae	GSI/ Eleutherocytes	Johansen, 1990
Dendrobium ellipsophyllum Tang & F.T.Wang		Dendrobiinae	GSI/ Eleutherocytes	Johansen, 1990
Dendrobium erostelle Seidenf.		Dendrobiinae	GSI/ Eleutherocytes	Johansen, 1990
Dendrobium falconeri Hook.		Dendrobiinae	GSI/ Eleutherocytes	Johansen, 1990
Dendrobium farmeri Paxton		Dendrobiinae	GSI/ Eleutherocytes	Johansen, 1990
Dendrobium formosum Roxb. ex Lindl.		Dendrobiinae	GSI/ Eleutherocytes	Johansen, 1990
Dendrobium gibsonii Paxton		Dendrobiinae	GSI/ Eleutherocytes	Johansen, 1990
Dendrobium gratiosissimum Rchb.f.		Dendrobiinae	GSI/ Eleutherocytes	Johansen, 1990
Dendrobium griffithianum Lindl.		Dendrobiinae	GSI/ Eleutherocytes	Johansen, 1990
Dendrobium hendersonii A.D.Hawkes & A.H.Heller		Dendrobiinae	GSI/ Eleutherocytes	Johansen, 1990
Dendrobium indivisum var. *pallidum* Seidenf.		Dendrobiinae	GSI/ Eleutherocytes	Johansen, 1990
Dendrobium infundibulum Lindl.		Dendrobiinae	GSI/ Eleutherocytes	Johansen, 1990
Dendrobium keithii Ridl.		Dendrobiinae	GSI/ Eleutherocytes	Johansen, 1990
Dendrobium lamellatum (Blume) Lindl.		Dendrobiinae	GSI/ Eleutherocytes	Johansen, 1990
Dendrobium leonis (Lindl.) Rchb.f.		Dendrobiinae	GSI/ Eleutherocytes	Johansen, 1990
Dendrobium lindleyi Steud.		Dendrobiinae	GSI/ Eleutherocytes	Johansen, 1990
Dendrobium linguella Rchb.f.		Dendrobiinae	GSI/ Eleutherocytes	Johansen, 1990
Dendrobium mannii Ridl.		Dendrobiinae	GSI/ Eleutherocytes	Johansen, 1990

Species	Subfamily	Subtribe	Self-incompatibility system	Reference
Dendrobium moschatum (Banks) Sw.		Dendrobiinae	GSI/ Eleutherocytes	Johansen, 1990
Dendrobium mucronatum Seidenf.		Dendrobiinae	GSI/ Eleutherocytes	Johansen, 1990
Dendrobium nathanielis Rchb.f.		Dendrobiinae	GSI/ Eleutherocytes	Johansen, 1990
Dendrobium pachyglossum C.S.P.Parish & Rchb.f.		Dendrobiinae	GSI/ Eleutherocytes	Johansen, 1990
Dendrobium pachyphyllum (Kuntze) Bakh.f.		Dendrobiinae	GSI/ Eleutherocytes	Johansen, 1990
Dendrobium panduriferum Hook.f.		Dendrobiinae	GSI/ Eleutherocytes	Johansen, 1990
Dendrobium parcum Rchb.f.		Dendrobiinae	GSI/ Eleutherocytes	Johansen, 1990
Dendrobium parishii H.Low		Dendrobiinae	GSI/ Eleutherocytes	Johansen, 1990
Dendrobium phalaenopsis Fitzg. (syn. *Dendrobium bigibbum* var. *superbum* Rchb.f.)		Dendrobiinae	GSI/ Eleutherocytes	Johansen, 1990
Dendrobium planibulbe Lindl.		Dendrobiinae	GSI/ Eleutherocytes	Johansen, 1990
Dendrobium podagraria Hook.f. (syn. *Dendrobium angulatum* Lindl.)		Dendrobiinae	GSI/ Eleutherocytes	Johansen, 1990
Dendrobium primulinum Lindl. (syn. *Dendrobium polyanthum* Wall. ex Lindl.)		Dendrobiinae	GSI/ Eleutherocytes	Johansen, 1990
Dendrobium pulchellum Roxb. ex Lindl.		Dendrobiinae	GSI/ Eleutherocytes	Johansen, 1990
Dendrobium secundum (Blume) Lindl. ex Wall.		Dendrobiinae	GSI/ Eleutherocytes	Johansen, 1990
Dendrobium senile C.S.P.Parish & Rchb.f.		Dendrobiinae	GSI/ Eleutherocytes	Johansen, 1990
Dendrobium setifolium Ridl.		Dendrobiinae	GSI/ Eleutherocytes	Johansen, 1990
Dendrobium subulatum (Blume) Lindl.		Dendrobiinae	GSI/ Eleutherocytes	Johansen, 1990
Dendrobium thyrsiflorum B.S.Williams		Dendrobiinae	GSI/ Eleutherocytes	Johansen, 1990
Dendrobium virgineum Rchb.f.		Dendrobiinae	GSI/ Eleutherocytes	Johansen, 1990
Dendrobium aggregatum Roxb. (syn. *Dendrobium lindleyi* Steud.)		Dendrobiinae	undescribed	Wilfret, 1968
Dendrobium arachnites Rchb.f. (syn. *Dendrobium dickasonii* L.O.Williams)		Dendrobiinae	undescribed	Wilfret, 1968
Dendrobium bullenianum Rchb.f.		Dendrobiinae	undescribed	Wilfret, 1968

Species	Subfamily	Subtribe	Self-incompatibility system	Reference
Dendrobium cariniferum Rchb.f.		Dendrobiinae	undescribed	Wilfret, 1968
Dendrobium chrysotoxum Lindl.		Dendrobiinae	undescribed	Wilfret, 1968
Dendrobium crumenatum Sw.		Dendrobiinae	undescribed	Wilfret, 1968
Dendrobium draconis Rchb.f.		Dendrobiinae	undescribed	Wilfret, 1968
Dendrobium farmeri Paxton		Dendrobiinae	undescribed	Wilfret, 1968
Dendrobium fimbriatum Hook.		Dendrobiinae	undescribed	Wilfret, 1968
Dendrobium formosum Roxb. ex Lindl.		Dendrobiinae	undescribed	Wilfret, 1968
Dendrobium leonis (Lindl.) Rchb.f.		Dendrobiinae	undescribed	Wilfret, 1968
Dendrobium linguella Rchb.f.		Dendrobiinae	undescribed	Wilfret, 1968
Dendrobium lituiflorum Lindl.		Dendrobiinae	undescribed	Wilfret, 1968
Dendrobium monile Kraenzl. (syn. *Dendrobium moniliforme* (L.) Sw.)		Dendrobiinae	undescribed	Wilfret, 1968
Dendrobium moschatum (Banks) Sw.		Dendrobiinae	undescribed	Wilfret, 1968
Dendrobium parishii H.Low		Dendrobiinae	undescribed	Wilfret, 1968
Dendrobium primulinum Lindl.		Dendrobiinae	undescribed	Wilfret, 1968
Dendrobium senile C.S.P.Parish & Rchb.f.		Dendrobiinae	undescribed	Wilfret, 1968
Dendrobium aphyllum (Roxb.) C.E.C.Fisch.		Dendrobiinae	undescribed	Huda and Wilcock, 2008
Dendrobium aphyllum (Roxb.) C.E.C.Fisch.		Dendrobiinae	undescribed	Niu et al., 2018
Dendrobium catenatum Lindl. (syn. *Dendrobium moniliforme* (L.) Sw.)		Dendrobiinae	GSI	Niu et al., 2018
Dendrobium chrysanthum Wall. ex Lindl.		Dendrobiinae	GSI	Niu et al., 2018
Dendrobium denneanum Kerr		Dendrobiinae	LSI	Niu et al., 2018
Dendrobium densiflorum Lindl.		Dendrobiinae	GSI	Niu et al., 2018
Dendrobium devonianum Paxton		Dendrobiinae	GSI	Niu et al., 2018

Species	Subfamily	Subtribe	Self-incompatibility system	Reference
Dendrobium farmeri Paxton		Dendrobiinae	undescribed	Niu et al., 2018
Dendrobium hancockii Rolfe		Dendrobiinae	GSI	Niu et al., 2018
Dendrobium jenkinsii Wall. ex Lindl.		Dendrobiinae	GSI	Niu et al., 2018
Dendrobium lindleyi Steud.		Dendrobiinae	GSI	Niu et al., 2018
Dendrobium longicornu Lindl.		Dendrobiinae	SSI (Shenzhen) and GSI (Malipo)	Niu et al., 2018
Dendrobium moniliforme (L.) Sw.		Dendrobiinae	GSI	Niu et al., 2018
Dendrobium polyanthum Wall. ex Lindl.		Dendrobiinae	undescribed	Niu et al., 2018
Dendrobium stuposum Lindl.		Dendrobiinae	undescribed	Niu et al., 2018
Dendrobium unicum Seidenf.		Dendrobiinae	GSI	Niu et al., 2018
Epidendrum cinnabarinum Salzm. ex Lindl.		Laeliinae	undescribed	East, 1940
Epipactis atrorubens (Hoffm.) Besser		Tribe Neottieae	undescribed	East, 1940
Erycina glossomystax (Rchb.f.) N.H.Williams & M.W.Chase		Oncidiinae	undescribed	Van der Cingel, 2001; Castro and Singer, 2019
Gomesa bifólia (Sims) M.W.Chase & N.H.Williams		Oncidiinae	undescribed	Torretta et al., 2011; Castro and Singer, 2019
Gomesa cf. *blanchetii* (Rchb.f.) M.W.Chase & N.H.Williams		Oncidiinae	undescribed	Pansarin et al., 2016; Castro and Singer, 2019
Gomesa cornigera (Lindl.) M.W.Chase & N.H.Williams		Oncidiinae	undescribed	Castro et al., 2022
Gomesa flexuosa (Lodd.) M.W.Chase & N.H.Williams		Oncidiinae	undescribed	Castro et al., 2022
Gomesa imperatoris-maximiliani (Rchb.f.) M.W.Chase & N.H.Williams (syn. Oncidium crispum Lodd.)		Oncidiinae	undescribed	East, 1940
Gomesa longicornu (Mutel) M.W.Chase & N.H.Williams (syn. *Oncidium unicorne* Lindl.)		Oncidiinae	undescribed	East, 1940

Species	Subfamily	Subtribe	Self-incompatibility system	Reference
Gomesa ranifera (Lindl.) M.W.Chase & N.H.Williams		Oncidiinae	undescribed	Castro et al., 2022
Gomesa riograndensis (Cogn.) M.W.Chase & N.H.Williams		Oncidiinae	undescribed	Castro et al., 2022
Gomesa varicosa (Lindl.) M.W.Chase & N.H.Williams		Oncidiinae	undescribed	Pansarin et al., 2016; Castro and Singer, 2019
Grandiphyllum divaricatum (Lindl.) Docha-Neto (syn. *Oncidium divaricatum* Lindl.)		Oncidiinae	undescribed	East, 1940; Tremblay et al., 2005
Ionopsis utricularioides (Sw.) Lindl.		Oncidiinae	undescribed	Aguiar and Pansarin, 2019;
Lepanthes woodburyana Stimson		Pleurothallidinae	undescribed	Tremblay et al., 2005
Liparis liliifolia (L.) Rich. ex Lindl.		Malaxidinae	undescribed	Whigham and O'Neil, 1991
Liparis makinoana Schltr.		Malaxidinae	undescribed	Oh et al, 2001
Lycaste aromatica (Graham) Lindl.		Maxillariinae	undescribed	Hietz et al., 2006
Lycaste cruenta (Lindl.) Lindl. (syn. *Maxillaria skinneri* Lindl.)		Maxillariinae	undescribed	East, 1940
Malaxis massonii (Ridl.) Kuntze		Malaxidinae	undescribed	Aragón and Ackerman, 2001
Masdevallia infracta Lindl.		Pleurothallidinae	SSI	Borba et al., 2011
Notylia barkeri Lindl.		Oncidiinae	undescribed	Singer and Koehler, 2003
Notylia longispicata Hoehne & Schltr.		Oncidiinae	undescribed	Singer and Koehler, 2003
Notylia nemorosa Barb.Rodr.		Oncidiinae	Eleutherocytes	Singer and Koehler, 2003
Notylia orbicularis A.Rich & Galeotti (syn. *Notylia tridachne* Lindl. & Paxton)		Oncidiinae	undescribed	Warford (1992)
Notylia trisepala Lindl. & Paxton		Oncidiinae	undescribed	Warford (1992)

Species	Subfamily	Subtribe	Self-incompatibility system	Reference
Octomeria crassifolia Lindl.		Pleurothallidinae	undescribed	Barbosa et al., 2013
Octomeria grandiflora Lindl.		Pleurothallidinae	undescribed	Barbosa et al., 2013
Oeoniella polystachys (Thouars) Schltr.		Angraecinae	undescribed	Agnew, 1986
Oncidium altissimum (Jacq.) Sw.		Oncidiinae	undescribed	Ackerman, 1995
Oncidium sphacelatum Lindl.		Oncidiinae	undescribed	East, 1940; Tremblay et al., 2005
Pelatantheria insectifera (Rchb.f.) Ridl.		Aeridinae	undescribed	Huda and Wilcock, 2008
Phalaenopsis equestris (Schauer) Rchb.f.		Aeridinae	undescribed	Cai et al., 2015
Phalaenopsis pulcherrima (Lindl.) J.J.Sm.		Aeridinae	undescribed	Agnew, 1986
Phalaenopsis schilleriana Rchb.f.		Aeridinae	undescribed	Agnew, 1986
Plectrelminthus caudatus (Lindl.) Summerh.		Angraecinae	undescribed	Agnew, 1986
Psychilis krugii (Bello) Sauleda		Laeliinae	undescribed	Ackerman, 1989
Psychilis krugii (Bello) Sauleda (syn. *Encyclia krugii* (Bello) Britton & P.Wilson)		Laeliinae	undescribed	Ackerman, 1989
Restrepia antennifera var. *gigante*a Kunth (syn. *Restrepia antennifera* Kunth)		Pleurothallidinae	GSI	Millner et al., 2015
Restrepia antennifera Kunth		Pleurothallidinae	GSI	Millner et al., 2015
Restrepia antennifera subsp. *hemsleyana* (Schltr.) H.Mohr (syn. *Restrepia antennifera* Kunth)		Pleurothallidinae	GSI	Millner et al., 2015
Restrepia aristulifera Garay & Dunst.		Pleurothallidinae	GSI	Millner et al., 2015
Restrepia brachypus Rchb.f. (genet 1)		Pleurothallidinae	GSI	Millner et al., 2015
Restrepia brachypus Rchb.f. (genet 3)		Pleurothallidinae	GSI	Millner et al., 2015
Restrepia guttulata Lindl. (genet 1)		Pleurothallidinae	GSI	Millner et al., 2015

Species	Subfamily	Subtribe	Self-incompatibility system	Reference
Restrepia sanguinea Rolfe		Pleurothallidinae	GSI	Millner et al., 2015
Restrepia condorensis Luer & R.Escobar		Pleurothallidinae	GSI	Millner et al., 2015
Restrepia mendozae Luer		Pleurothallidinae	GSI	Millner et al., 2015
Restrepia cuprea Luer & R.Escobar		Pleurothallidinae	GSI	Millner et al., 2015
Restrepia falkenbergii Rchb.f.		Pleurothallidinae	GSI	Millner et al., 2015
Restrepia muscifera (Lindl.) Rchb.f. ex Lindl.		Pleurothallidinae	GSI	Millner et al., 2015
Restrepia purpurea Luer & R.Escobar		Pleurothallidinae	GSI	Millner et al., 2015
Restrepia citrina Luer & R.Escobar		Pleurothallidinae	GSI	Millner et al., 2015
Restrepia seketii Luer & R.Escobar		Pleurothallidinae	GSI	Millner et al., 2015
Restrepia vasquezii Luer		Pleurothallidinae	GSI	Millner et al., 2015
Restrepia wageneri Rchb.f.		Pleurothallidinae	GSI	Millner et al., 2015
Restrepia dodsonii Luer		Pleurothallidinae	GSI	Millner et al., 2015
Restrepia mohrii Braem		Pleurothallidinae	GSI	Millner et al., 2015
Restrepia iris Luer		Pleurothallidinae	GSI	Millner et al., 2015
Restrepia elegans H.Karst.		Pleurothallidinae	GSI	Millner et al., 2015
Restrepia cloesii Luer		Pleurothallidinae	GSI	Millner et al., 2015
Restrepia contorta (Ruiz & Pav.) Luer		Pleurothallidinae	GSI	Millner et al., 2015
Restrepia howei Luer		Pleurothallidinae	GSI	Millner et al., 2015
Rodriguezia bahiensis Rchb.f.		Oncidiinae	undescribed	Carvalho and Machado, 2006
Rodriguezia decora (Lem.) Rchb.f.		Oncidiinae	undescribed	Pansarin et al., 2018
Rodriguezia granadensis (Lindl.) Rchb.f.		Oncidiinae	GSI	Ospina-Calderón et al., 2015
Rodriguezia lanceolata Ruiz & Pav.		Oncidiinae	undescribed	Pansarin et al., 2018

Species	Subfamily	Subtribe	Self-incompatibility system	Reference
Sobennikoffia humbertiana H. Perrier		Angraecinae	undescribed	Agnew, 1986
Stelis argentata Lindl.		Pleurothallidinae	undescribed	Christensen, 1992
Tolumnia guibertiana (A.Rich.) Braem		Oncidiinae	undescribed	Vale et al., 2011
Tolumnia lemoniana (Lindl.) Braem (syn. *Oncidium lemonianum* Lindl.)		Oncidiinae	undescribed	East, 1940
Tolumnia variegata (Sw.) Braem		Oncidiinae	undescribed	Ackerman and Montero- Oliver, 1985
Trichocentrum ascendens (Lindl.) M.W.Chase & N.H.Williams (syn. *Oncidium ascendens* Lindl.)		Oncidiinae	undescribed	Parra-Tabla et al., 2000
Trichocentrum cavendishianum (Lindl.) M.W.Chase & N.H.Williams (syn. *Oncidium cavendishianum* Bateman)		Oncidiinae	undescribed	East, 1940
Trichocentrum luridum (Lindl.) M.W.Chase & N.H.Williams (syn. *Oncidium cosymbephorum* C.Morren)		Oncidiinae	undescribed	Carmona-Díaz and García-Franco, 2009; Cen, 2016
Trichocentrum microchilum (Bateman ex Lindl.) M.W. Chase & N.H.Williams		Oncidiinae	undescribed	East, 1940
Trichocentrum pumilum (Lindl.) M.W.Chase & N.H.Williams		Oncidiinae	undescribed	Pansarin and Pansarin, 2011

TABLE S2. Vouchers of sampled specimens of *Brasiliorchis* R.B.Singer, S.Koehler & Carnevali deposited at herbarium UEC (State University of Campinas/UNICAMP, Brazil) and used in this study.

Accession (UEC)	Species	Country	State	City
205928	*B. phoenicanthera* (Barb.Rodr.) R.B. Singer, S. Koehler & Carnevali	Brazil	Unknown	Unknown
205914			São Paulo	Bertioga
204012			São Paulo	Brotas
205258			São Paulo	Near Cananeia
38869			São Paulo	Near Cananeia
203107			São Paulo	Unknown
203075			Minas Gerais	Jacinto
203096			São Paulo	São Paulo
203097			Paraná	Guaíra
203100			Unknown	Unknown
203103			Minas Gerais	Baependi
203101			São Paulo	Santo André
203099			Santa Catarina	Matos Costa
203098			Santa Catarina	Matos Costa
203985			Bahia	Itamarajú
204007			Paraná	Jaguariaiva
204006			São Paulo	Apiaí
205933			São Paulo	São Bernardo do Campo
204005			Paraná	Jaguariaiva
203939	*B. picta* (Hook.) R.B. Singer, S. Koehler & Carnevali	Brazil	São Paulo	Ubatuba
203940			São Paulo	Teodoro Sampaio
203941			São Paulo	Teodoro Sampaio
203942			São Paulo	São Paulo
203943			São Paulo	Teodoro Sampaio
203944			São Paulo	Santo André
203945			Minas Gerais	Camanducaia
203947			São Paulo	Brotas
203948			São Paulo	Campos do Jordão
203949			São Paulo	Ubatuba
203950			Minas Gerais	Camanducaia

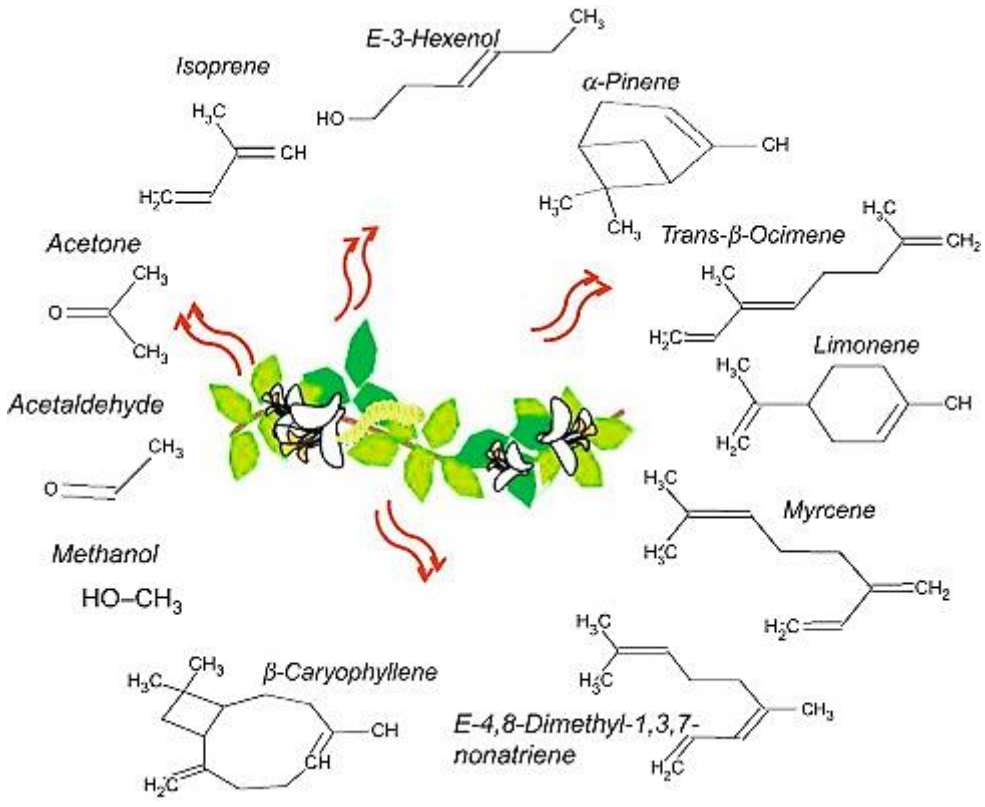
Accession (UEC)	Species	Country	State	City
203951			São Paulo	Terra Nova
203952			Minas Gerais	Camanducaia
203953			São Paulo	Apiaí
203954			São Paulo	Apiaí
205486			São Paulo	Campos do Jordão
204014			São Paulo	Camanducaia
203946			Minas Gerais	Camanducaia
203956			São Paulo	Embu-Guaçu
203957			São Paulo	Campos do Jordão
203958			São Paulo	Campos do Jordão
203959			São Paulo	São Bernardo do Campo
203960			São Paulo	Apiaí
205484			Minas Gerais	Jacinto
203961			São Paulo	Apiaí
203980			Rio de Janeiro	Parati
203979			Rio de Janeiro	Parati
203978			São Paulo	Santos
205483			Mato Grosso do Sul	Bataguassu
203977			Minas Gerais	Caldas
203955			São Paulo	Embu-Guaçú
205172			São Paulo	Embu-Guaçú
205972			São Paulo	Embu-Guaçú
205232			São Paulo	Embu-Guaçú
204023			São Paulo	Cunha
205944			São Paulo	Brotas
205916			São Paulo	Santo André
203963			São Paulo	Brotas
203962			São Paulo	Brotas
205133			São Paulo	Cunha
205931			São Paulo	Santo André
204023			São Paulo	Cunha
205232			São Paulo	Embu-Guaçú
203975			São Paulo	Apiaí
205920			São Paulo	Campos do Jordão

Accession (UEC)	Species	Country	State	City
203976			São Paulo	Apiaí
203973			São Paulo	Apiaí
205254			Paraná	Jaguariaíva
203971			São Paulo	Campos do Jordão
205949	*B. porphyrostele* (Rchb.f.) R.B. Singer, S. Koehler & Carnevali	Brazil	Santa Catarina	Blumenau
203993			Rio de Janeiro	Nova Friburgo
203992			Santa Catarina	Terra Nova
204026			Santa Catarina	Matos Costa
203989			Santa Catarina	Matos Costa
205187			São Paulo	Itanhaém
203996			São Paulo	Teodoro Sampaio
203997			São Paulo	São Paulo
205131			Santa Catarina	São Joaquim
203994			São Paulo	Brotas
203987			Santa Catarina	Terra Nova
205186			Santa Catarina	Terra Nova
203998			Santa Catarina	Vacas Gordas and Urubici
203999			Santa Catarina	Terra Nova
204000			Santa Catarina	Matos Costa
203927			Santa Catarina	Terra Nova
203928			Santa Catarina	Terra Nova
205134			Santa Catarina	Matos Costa
203930			Santa Catarina	Lages
205487			Paraná	Jaguariaiva
203931			Santa Catarina	Lages
203932			Santa Catarina	Lages
205490			Unknown	Unknown
205488			Santa Catarina	Terra Nova
203933			Santa Catarina	Lages
203934			São Paulo	Santo André
203935			Santa Catarina	Lages
205489			Santa Catarina	Blumenau
203936			São Paulo	Santo André

CHAPTER II

Interespecific plasticity of floral volatile organic compounds in *Brasiliorchis* orchids

(This chapter is formatted according to the journal Phytochemistry Letters)



ABSTRACT

Floral fragrances are complex bouquets of specific volatile organic compounds (VOCs) in which each chemical compound varies in proportion. Floral chemical profiles of orchid species from the Brazilian Atlantic Forest were here investigated. Our goals were: (1) to describe floral chemical profiles of four new species within *Brasiliorchis* (*B. barbozae*, *B. phoenicanthera*, *B. porphyrostele* and *B. schunkeana*) and report new data on four previously studied species (*B. gracilis*, *B. marginata*, *B. picta* and *B. ubatubana*). Furthermore, we chose these species, (2) to compare four species previously sampled by Flach et al. (2004) through another technique of collecting floral VOCs, and also sampling of floral VOCs in four unpublished species in the literature.We studied eight species of *Brasiliorchis* (*B. barbozae*, *B. gracilis*, *B. marginata*, *B. phoenicanthera*, *B. picta*, *B. porphyrostele*, *B. schunkeana* and *B. ubatubana*). Floral VOCs were assessed using techniques of headspace collection and GC-MS with thermal desorption. Volatile compounds varied from 34 in *B. marginata* and *B. ubatubana* to 70 in *B. barbozae* and *B. picta*. Floral VOCs are largely dominated by alcohol, alkenes, alkanes, aldehydes and monoterpenes and aromatic compounds. *Brasiliorchis* species have very different floral chemical profiles, in which only 15.4% of floral VOCs were shared between two or more species. Negative frequency selection acting on floral traits of food deceptive flowers or drift may explain high diversity of floral VOCs among *Brasiliorchis* species.

KEYWORDS

Brazilian Atlantic Forest, chemical ecology, GC-MS, Maxillariinae, Orchidaceae.

INTRODUCTION

Orchids are one of the most species-rich families of angiosperms (World Checklist of Selected Plant Families, 2017) with about 25,000 species described (Dressler, 1993; Chase et al., 2015). The great variation of floral morphology in the family has been attributed to the diversity of mechanisms to ensure reproductive success (Van der Cingel, 2001; Cozzolino et al., 2005; Micheneau et al., 2009). One consequence of such diversity is a great variety of floral fragrances.

Intercommunication between plants and pollinators often comes from floral fragrances (Jersáková et al., 2006). Floral fragrances are complex bouquets of specific volatile organic compounds (VOCs), in which each chemical compound varies in proportion rates and on the pollinator behavior (Van der Niet et al., 2014). Previous studies suggested that olfactory sensory mechanisms and behavioral preferences of pollinators are associated with the evolution of floral VOCs composition (Jürgens et al., 2013). Therefore, knowledge on these chemical compounds is crucial to understand ecology and evolution of orchid-pollinator relationships (Schiestl, 2015). The morphological and chemical floral diversity in orchids is notorious (Mondragón-Palomino and Theissen, 2009) and is often essential in the reproductive isolation of sympatric species (Schiestl and Schlüter, 2009). Recent studies have shown that color and floral fragrance in Orchidaceae species may be equally important in relation to pollinators (Klahre et al., 2011), but unlike color variation, the variability of volatiles has received limited attention (Dormont et al., 2010; Delle-Vedove et al., 2011).

Maxillariinae is one of the most diverse orchid subtribes in the neotropics, with approximately 600 recognized species (Whitten et al., 2007). *Brasiliorchis* is a neotropical genus that occurs in the Brazilian Atlantic Forest and surrounding vegetation from the States of Rio Grande do Sul to Bahia, extending to Misiones (Argentina) (Singer et al., 2007; Pridgeon et al., 2009). Currently, 15 species are recognized to the genus, and they can be easily identified by their bifoliated and furrowed pseudobulbs, conduplicate leaves and roots varying from whitish to reddish in color. Flowers are campanulate to spreading and long-lasting, showy, honey-scented, rewardless, and usually yellowish or creamy in color (Singer et al., 2007; Pridgeon et al., 2009).

Kaiser (1993) was the first author to describe chemical profiles of floral volatiles considering genus *Brasiliorchis* (*B. picta*), in which the main VOC found was estragole (42.5%). Later, Flach et al. (2004) identified the VOCs of five species of

Brasiliorchis (*B. chrysantha*, *B. gracilis*, *B. marginata*, *B. picta* and *B. ubatubana*) and found high interspecific diversity, in which the main VOC found was linalool (up to 70.5%). Although the research by Flach et al. (2004) contributed a lot to the knowledge about the variation of VOCs in *Brasiliorchis*, they only sampled part of the genus diversity.

Specifically, for the subtribe Maxillariinae, which contains about 600 Neotropical species, knowledge about reproductive systems as well as about patterns of plasticity in floral VOCs has been little explored. Therefore, this study model is interesting, since the approach of the floral VOCs and the reproductive system within the genus could elucidate and contribute to the greater knowledge of this group (i.e. if the species are related and difficult to identify or if they are sister species or form complexes of species), ecology and conservation of these species. Among the subtribes of Orchidaceae with studies concerning floral VOCs, Maxillariinae has few available reports on intra- and interspecific plasticity of floral VOCs. Thus, the determination of the chemical composition and the diversification pattern of VOCs, in *Brasiliorchis* species, can result in informative characters to identify reproductively isolated lineages.

In order to fill in this gap, our aims were: (1) to describe floral chemical profiles of four new species within *Brasiliorchis* (*B. barbozae*, *B. phoenicanthera*, *B. porphyrostele* and *B. schunkeana*) and report new data on four previously studied species (*B. gracilis*, *B. marginata*, *B. picta* and *B. ubatubana*). Furthermore, we chose these species, (2) to compare four species previously sampled by Flach et al. (2004) through another technique of collecting floral VOCs, and also sampling of floral VOCs in four unpublished species in the literature.

MATERIAL AND METHODS

Sampling

We sample one or two individuals per species (Table 1). Flowers at anthesis were analyzed from plants cultivated in the nursery orchid collection of the Núcleo de Pesquisa Orquidário/Orquidário Dr. Frederico Carlos Hoehne (IPA, Instituto de Pesquisas Ambientais previously IBOT, Instituto de Botânica, São Paulo, Brazil). Plants of *B. schunkeana* were acquired from Orquidário Colibri, São Lourenço da Serra, São Paulo,

Brazil. Vouchers of all sampled species were deposited at the Herbarium of State University of Campinas/UNICAMP, Brazil (UEC) (Table 1).

Floral chemical profiles analyses were performed at Laboratório de Interação Animal-Planta (LABIAP) located at IPA, Instituto de Pesquisas Ambientais previously IBT, Instituto de Botânica, São Paulo, Brazil. The samples were collected on the first day of anthesis (because VOCs are constantly degraded during floral lifespan), from 9 a.m. to 5 p.m., considering sampling periods of 30 min to 3 h (Table 1). We used different sampling intervals, because the retention of key-compounds (mainly monoterpenes and sesquiterpenes) was different for each species sampled. Flowers were kept apart from their vegetative constituents in plastic bags with side vents for air input and output, ensuring the maintenance of gas exchange (Cardoso-Gustavson et al., 2017). VOCs of all sampled species were collected through cartridges containing 100 mg of TENAX-TA adsorbents (Supelco, mesh 60/80), with one end fixed in a bag opening and the other connected to a suction pump, ensuring the passage of the air inside the cartridge. We used an artificial light support above the floral headspace system as volatiles are released in the presence of sunlight. The samples were collected for majoring compounds for plastic bags. Values obtained from VOCs were subtracted from blank samples (Cardoso-Gustavson et al., 2017). Flows of the sampled air and the air inserted into the bags were around 0.225 L/min^{-1}. Volatile compounds were analyzed by gas chromatography-mass spectrometry (GC-MS) (MSD 5973; Agilent GC 7890B). Trapped compounds were desorbed with a thermal desorption unit (Perkin-Elmer ATD400 Automatic Thermal Desorption system (Perkin Elmer, Waltham, MA, USA) at 250°C for 10 min, cryofocused at -30 °C and injected onto an HP-5 capillary column (50 m × 0.2 mm i.d. × 0.5 µm film thickness; Hewlett-Packard) with helium as a carrier gas. The oven temperature program was held at 40 °C for 1 min and then raised to 210 °C at a rate of 5 °C min^{-1}, and finally further to 250 °C at a rate of 20 °C min^{-1}. We used the technique with a thermal desorption system (ATD) which is more effective in preventing early degradation of floral VOCs than elution of floral VOCs in a liquid substrate (hexane or similar) (SPME). Some authors showed that degradation of polymer polyamide 6,6 occurs earlier in the SPME method when compared to other methodologies. Thus, polymer degradation may alter quantity and type of extracted VOCs. In this regard, the literature corroborates the efficiency of the system used here.

TABLE 1. Specimens of *Brasiliorchis* sampled in this study, sampling time and interval. UEC = Herbarium of State University of Campinas (UNICAMP); IBT = Orchid nursery of the Institute of Botany of São Paulo.

Species	Collection site	Accession number	Date and sampling time	Sampling interval (min)
Brasiliorchis barbozae	Santana do Riacho, MG	UEC205925/ IBT12732	20apr2019/ 9:06 a.m. – 12:06 p.m.	180
	São Bernardo do Campo, SP	UEC202753/ IBT18222	20apr2019/ 9:15 a.m. – 12:15 p.m.	180
Brasiliorchis gracilis	Matos Costa, SC	UEC203136/ IBT4695	26jul2019/9:45 a.m. – 11:45 a.m.	120
	Terra Nova, SC	UEC204026/ IBT12068	26jul2019/9:30 a.m. – 11:30 a.m.	120
Brasiliorchis marginata	Cunha, SP	UEC203070/ IBTP5882	05oct2017/2:50 p.m. – 4:20 p.m.	90
Brasiliorchis phoenicanthera	Terra Nova, SC	UEC203098/ IBT1848	31jul2017/1:52 p.m. – 2:52 p.m.	60
	Matos Costa, SC	UEC203928/ IBT6113	31jul2017/1:32 p.m. – 2:32 p.m.	60
Brasiliorchis picta	Embu-Guaçú, SP	UEC205986/ IBT3692	31jan2018/2:48 p.m. – 3:18 p.m.	30
	Santo André, SP	UEC202754/ IBT9724	05jun2018/2:20 p.m. – 2:50 p.m.	30
Brasiliorchis porphyrostele	São Joaquim, SC	UEC205131/ IBT4993	01aug2017/10:55 a.m. – 12:25 p.m.	90
Brasiliorchis schunkeana	Orquidário Colibri (specimen 1)	in cultivation at UNICAMP	12apr2019/1:59 p.m. – 4:59 p.m.	180
	Orquidário Colibri (specimen 2)	in cultivation at UNICAMP	12apr2019/9:10 a.m. – 12:10 p.m.	180
Brasiliorchis ubatubana	Camanducaia, MG	UEC205947/ IBT1099D	29apr2019/1:40 p.m. – 4:10 p.m.	150

Data analysis

Chromatograms containing floral chemical profiles identification were based on the library NIST® (National Institute of Standards and Technology) of software MSD ChemStation F.01.00.1903® (MS HP, USA) (Table 2). We used absolute peak areas to

calculate the percentage of each compound in the sample. We calculated the percentage by comparing the sum of peak areas (a hundred percent of compounds) and the individual area of each compound. We defined optimal collection times for each species considering the maximum number of peaks with a similarity of mass spectra higher than 80%, after checking if VOCs are produced by flowers according to NIST®, PubChem® and Pherobase® libraries and previously published studies. We performed these steps at "Laboratório de Interação Animal-Planta (LABIAP)" and "Núcleo de Ecologia Aquática e Terrestre", Instituto de Botânica de São Paulo, São Paulo, Brazil.

RESULTS

Table 2 summarizes VOCs detected from flower headspace of each eight sampled species of *Brasiliorchis.* A total of 238 VOCs were identified in our study. Volatile compounds varied from 34 in *B. marginata* and *B. ubatubana* to 70 in *B. barbozae* and *B. picta* (Table 2). Figure 1 indicates main floral volatile categories for *Brasiliorchis* species sampled in this study. Considering the eight species sampled, only four of 26 compounds (15.4%) are shared between two or more species: Hexadecane, Cetene, ρ-anisaldehyde and 1-hexanol, 2-ethyl. Floral VOCs of species *B. barbozae*, *B. marginata* and *B. schunkeana* are largely dominated by alcohol compounds (Hexanoic acid, Octanoic acid, 1-hexanol, 2-ethyl e *n*-tetracosanol-1). Alkenes (E-15-heptadecenal) and alkanes (Hexadecane) were also identified as main floral volatiles in *B. barbozae*. Besides *B. barbozae,* alkene was detected as an important compound category in *B. picta* (37.48% of E-14-hexadecenal). Alkanes (Tetradecane, Pentadecane, Tridecane) and aldehydes (Nonanal, Decanal, Undecanal) dominated floral volatiles of *B. porphyrostele*. Floral volatiles of *B. gracilis* were mainly represented by monoterpene β-Ocimene. Monoterpenes were also representative in *B. picta* and *B. phoenicanthera* (Carveol), *B. barbozae* and *B. ubatubana* (both Estragole). Aromatic volatiles also emerged as representative compounds in floral volatiles of *B. phoenicanthera, B. schunkeana* and *B. ubatubana* (Phenylethyl alcohol and, only in the latter, ρ-anisaldehyde) (Table 2).

Among the VOCs identified in *Brasiliorchis*, there are some classes of volatiles that were less representative in the composition of floral volatiles, but are equally important in the ecological relationships between animals and plants. These floral VOCs included: Tetrachlorethylene - an amine identified in *B. barbozae* (0.02%) and *B. picta* (0.07%); 6,19-Icosadiene - an alkadiene identified in *B. barbozae* (1.39%); Dodecanoic

acid and Methyl-*n*-tridecanoate - fatty acids identified in *B. gracilis* (0.09% and 0.37%), respectively; Ethyl-2-butylnoate - an alkyne identified in *B. picta* (0.91%); Methyl salicylate – an ester identified only by Flach et al. (2004) in *B. marginata* (0.6%) (Table 2). Below, we also compared data about floral volatiles between the four species in common to both studies:

B. gracilis: Only our study found the monoterpene β-ocimene that represented a high percentage (73.49%). Our study also found an abundance of alcohols, aldehydes, alkanes and alkenes (48 volatiles), while Flach et al. (2004) found only three. Also, we found only one ketone (Ethanone, 1-(2-methyl-2-cyclopenten-1-yl)- (0.02%) against three found by Flach et al. (2004). Only Flach et al. (2004) found the aldehydes *n*-Decanal, *n*-Nonanal and *n*-Tetradecane (4.9%, 3.0% and 0.7%), respectively. Flach et al. (2004) found more terpenoids (mono- and sesquiterpene), prevailing *trans*-β-Ocimene (30.3%) (23 against 11 volatiles) (Table 3).

B. marginata: Only our study found the alcohols 1-Octanol and Octanoic acid that represented a high percentage (27.26% and 36.43%), respectively. Our study also found an abundance of alcohols, aldehydes and alkanes (15 volatiles), while Flach et al. (2004) found only four. Also, we found only one ketone (Sulcatone) (0.37%) against three found by Flach et al. (2004). Such as *B. gracilis*, only Flach et al. (2004) found the aldehydes *n*-Decanal and *n*-Nonanal (4.4% and 1.9%), respectively. Flach et al. (2004) found more terpenoids, prevailing the monoterpenes α-Copaene (9.6%) and Limonene (9.0%) (22 against 15 volatiles) (Table 4).

B. picta: Our study found a great diversity of volatiles to the species compared to Flach et al. (2004) (total of 69 against 9), prevailing the alcohols, aldehydes, alkanes, alkenes, aromatics and terpenoids volatile classes (63 against 6). Only our study found alkyne and amine volatiles. The volatiles more abundant in our study were the alkenes E-14-Hexadecenal (37.48%) and E-15-Heptadecenal (9.32%). Flach et al. (2004) found a great percentage of the monoterpene Linalool (70.5%), being a significative volatile to the species (Table 5).

B. ubatubana: Our study found twice the number of volatiles compared to Flach et al. (2004) (34 against 16). Only our study found the alcohol Hexanoic acid (10.58%). The most abundant volatiles in both studies were the aromatic ρ-anisaldehyde (22.90%) and Phenylethyl alcohol (16.35%) (our study) and Phenylacetaldehyde (47.5%) and Phenylethyl alcohol (35.7%) (Flach et al., 2004). Of these, the volatile Phenylethyl

alcohol was identified in both collection methodologies. Among the terpenoids, *cis*-Linalool and α-Copaene were the only ones to coincide in both studies (Table 6).

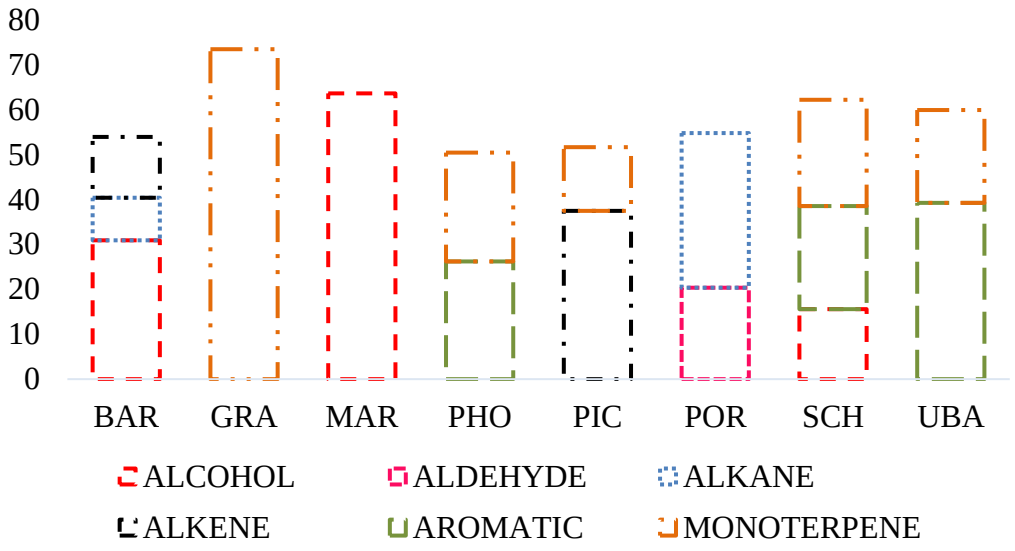


FIGURE 1. Relative frequency of >50% volatile organic compound (VOC) categories for each *Brasiliorchis* species sampled. BAR = *B. barbozae* (Loefgr.) R.B.Singer, S.Koehler & Carnevali, GRA = *B. gracilis* (Lodd.) R.B.Singer, S.Koehler & Carnevali, MAR = *B. marginata* (Lindl.) R.B.Singer, S.Koehler & Carnevali, PHO = B. *phoenicanthera* (Barb.Rodr.) R.B.Singer, S.Koehler & Carnevali, PIC = *B. picta* (Hook.) R.B.Singer, S.Koehler & Carnevali, POR = *B. porphyrostele* (Rchb.f) R.B.Singer, S.Koehler & Carnevali, SCH = *B. schunkeana* (Campacci & Kautsky) R.B.Singer, S.Koehler & Carnevali, UBA = *B. ubatubana* (Hoehne) R.B.Singer, S.Koehler & Carnevali.

TABLE 2. Relative abundances of volatile organic compounds identified from the floral headspace of *Brasiliorchis* species. Unidentified VOCs are volatiles that were obtained in TENAX-TA adsorbent or plastic bags of floral headspace system. For interspecific variation of floral volatiles were used n=2 specimens in *B. barbozae*, *B. gracilis*, *B. phoenicanthera*, *B. picta* and *B. schunkeana*. For *B. marginata*, *B. porphyrostele* and *B. ubatubana* was used n=1 specimen (see Tab. 1 of this chapter). BAR = *B. barbozae* (Loefgr.) R.B.Singer, S.Koehler & Carnevali, GRA = *B. gracilis* (Lodd.) R.B.Singer, S.Koehler & Carnevali, MAR = *B. marginata* (Lindl.) R.B.Singer, S.Koehler & Carnevali, PHO = B. *phoenicanthera* (Barb.Rodr.) R.B.Singer, S.Koehler & Carnevali, PIC = *B. picta* (Hook.) R.B.Singer, S.Koehler & Carnevali, POR = *B. porphyrostele* (Rchb.f) R.B.Singer, S.Koehler & Carnevali, SCH = *B. schunkeana* (Campacci & Kautsky) R.B.Singer, S.Koehler & Carnevali, UBA = *B. ubatubana* (Hoehne) R.B.Singer, S.Koehler & Carnevali.

Compound	BAR	GRA	MAR	PHO	PIC	POR	SCH	UBA
ALCOHOL								
11-Methyldodecanol				0.31				
1-Dodecanol		1.14				2.34		
1-Dodecanol, 3,7,11-trimethyl-	0.43	1.04			0.12			
1-Hexanol, 2-ethyl-	21.56	12.44		15.34	3.58			
1-Octanol			27.26					
1-Tetradecanol					1.15			
3-Hexen-1-ol							0.22	
Cyclohexanol, 3,5-dimethoxy		0.10						
E-2-Hexadecacen-1-ol		0.22						
Hexanoic acid		0.13					15.58	10.58
Hexanoic acid, 2-ethyl-		0.41						
Methylene chloride							1.02	
η-Decanoic acid						1.49		
Nonanoic acid		0.06		1.05				
η-Tetracosanol-1	9.36							
Octanoic acid			36.43	1.27				
α-Cumyl alcohol	1.04							

Compound	BAR	GRA	MAR	PHO	PIC	POR	SCH	UBA
ALDEHYDE								
(Z,Z)-3,6-nonadienal		0.37						
1-Hexadecanol					0.73	0.05		
1-Hexadecanol, 2-methyl-	3.04				1.86			
1-Nonanol						0.06		
1-Octanol, 3,7-dimethyl-						0.32		
2-Decenal, (E)-		0.08	0.06			0.67		
2-Dodecanone						0.54		
2-Ethyl-1-dodecanol				0.17				
2-Methyl-4-pentenal							0.02	
2-Undecenal				0.23	4.09			
3-Methyl-3-cyclohexen-1-one					0.19			
4-Nonenal, (E)-								0.09
Butanal			0.07					
cis-3-Hexenol								0.25
Decanal		1.16	1.40	2.84		6.83		
Dodecanal	0.51	0.41		1.83	1.94		11.39	3.39
Heptanal	0.41		0.23		0.25			
Hexanal	0.17	0.38					0.04	0.07
Lilial		0.16						
Nonanal		0.51	2.43	2.06		7.96		
Octanal		0.40	0.49					
Tetradecanal				5.38				
Undecanal					1.93	5.60		
Undecanal, 2-methyl-				0.11				
Z-10-Pentadecen-1-ol					0.41			
ALKADIENE								
6,19-Icosadiene	1.39							
ALKANE								
1-Iodo-dodecane				0.09				

Compound	BAR	GRA	MAR	PHO	PIC	POR	SCH	UBA
2-Hexadecanol					0.69			
2-Methyloctadecane	1.87							
3-Eicosane, (E)-	0.78							
3-Methyleicosane					1.86			
4-Ethylundecane	0.10							
4-Methylnonane	0.02							
5,8-Diethyldodecane	1.82							
Cyclododecane		0.03						
Cyclopentadecane				1.23				
Decane, 2,3,5-trimethyl-			0.26	0.33	0.16			
Decane, 2,3,7-trimethyl-								
Decane, 2,3,8-trimethyl-								
Decane, 2,4,6-trimethyl-	0.72							
Decane, 3-methyl-	0.25							
Decylcyclohexane						0.57		
Dodecane					3.61			
Dodecane, 2,6,10-trimethyl-		0.09			0.08			
Dodecane, 2,6,11-trimethyl-	0.43	0.17		0.31	0.13		1.40	
Dodecane, 2,7,10-trimethyl-		0.11	0.47					0.02
Dodecane, 3-methyl-				0.73				
Dodecane, 4,6-dimethyl-	0.41							
Dodecane, 4-methyl-				0.77				
Dodecane, 5,8-diethyl-		0.23						
Eicosane, 10-methyl-						1.48		
Heptacosane						0.32		
Heptadecane	5.15	1.30	0.53		5.26	1.09		
Heptadecane, 2,6,10,14-tetramethyl-				2.51	2.64	3.13		
Heptadecane, 2,6,10,15-tetramethyl-	2.01	0.03						
Heptadecane, 8-methyl-						2.41		
Heptane, 5-ethyl-2-methyl-	0.42				0.11			
Hexadecane	9.48	4.93	2.99	0.55	3.76			
Hexadecane, 2,6,10,14-tetramethyl-	0.21	0.48			0.31			

Compound	BAR	GRA	MAR	PHO	PIC	POR	SCH	UBA
Hexadecane, 2,6,11,15-tetramethyl-						0.74		
Nonane		0.26					0.18	
Nonane, 2,6-dimethyl					0.03			
Nonane, 2-Methyl-5-propyl-				0.60				
Nonane, 5-butyl-						1.61		
Octadecane						0.60		
Octadecane, 1-chloro				0.70				
Octadecane, 2-methyl-		0.03						
Octadecane, 3-ethyl-5-(2-ethylbutyl)-		0.27						
Octadecane, 5,14-dibutyl-						0.71		
Octane		0.20		0.47			0.29	
Octane, 5-ethyl-2-methyl-				0.41		0.49		
Pentacosane, 13-phenyl-		0.20						
Pentadecane				10.59		11.41		
Pentadecane, 2,6,10,14-tetramethyl-		0.20						
Pentadecane, 2,6,10-trimethyl-		0.16						
Pentadecane, 2-methyl-	1.65	0.21		2.48	0.54	2.80		
Pentadecane, 3-methyl-	1.15	0.23		1.04	0.35	2.66		
Pentane, 2,3,4-trimethyl-		0.00						
tert-Hexadecanethiol					0.55			
Tetradecane		1.12	0.72	1.89	8.64	14.45		
Tetradecane, 2,6,10-trimethyl-		0.21						
Tetradecane, 3-methyl-						1.26		
Tetradecane, 4- methyl-				0.34		0.60		
trans-phyt-2-ene	1.30	0.15			1.44			
Tridecane	0.30	0.23	0.54		1.68	8.57		
Tridecane, 2-methyl-	0.40		0.89		0.24			
Tridecane, 3-ethyl-		0.05						
Undecane					0.18	0.69		
Undecane, 2,6-dimethyl-					0.36			
Undecane, 2,8-dimethyl-						0.02		
Undecane, 2,9-dimethyl-		0.13						

Compound	BAR	GRA	MAR	PHO	PIC	POR	SCH	UBA
Undecane, 3,7-dimethyl-		0.22						
Undecane, 4,7-dimethyl-					0.15			
Undecane, 4,8-dimethyl-	0.95				0.58			
Undecane, 4-ethyl-	0.46							
Undecane, 4-phenyl-	0.46							
Undecane, 5,7-dimethyl-	0.47				0.29			
Undecane, 5-phenyl-	1.04							
ALKENE								
11-Tricosene	0.60				0.37			
1-Docosene					2.17			
1-Pentadecene	1.33				0.57			
1-Tetradecene	1.01							
1-Tridecene	1.28							
2,4,6,8-Tetramethyl-1-undecene		0.04						
2-Methyl-E-7-Hexadecene		0.41						
3-Octadecene, (E)-		0.44						
5-Eicosene, (E)-	4.72				2.89			
5-Eicosene, (E)- 7-Tetradecene						0.38		
8-Heptadecene	2.99	0.34			2.40			
Cetene	4.85	0.72			3.87			
E-14-Hexadecenal		3.09			37.48			
E-15-Heptadecenal	13.59				9.32			
trans-3-Octene	1.71							
trans-9-Octadecene	3.85							
ALKYNE								
Ethyl 2-butynoate					0.91			

Compound	BAR	GRA	MAR	PHO	PIC	POR	SCH	UBA
AMINE								
Tetrachloroethylene	0.02				0.07			
AROMATIC								
Alloaromadendrene							0.20	
Anethole							0.05	
Aromadendrene								0.17
Benzaldehyde			0.79	1.25			0.12	
Benzeneacetaldehyde				4.09	5.42		8.74	
Benzocyclobutene	2.14							
Benzoic Acid			0.31	14.46		1.58		
Benzyl Isovalerate							5.73	
Benzyl-2-Methylbutirate-							3.18	
Benzylalcohol	3.14							
Cinnamaldehyde, (E)-							1.36	4.78
cis-3-Hexenyl Isovalerate							0.10	
Ethylbenzene	0.29							
Indole				1.05				
Indole-2,3-dione								0.75
Mesitylene	0.23				0.13			
o-Cymene							1.11	
o-Guaiacol							0.07	
ρ-Anisaldehyde					8.71			22.90
Phenol						0.05		
Phenylethyl Alcohol	6.14		1.06	26.24	2.93		22.96	16.35
ρ-tert-butylphenol		0.13			0.18			
Toluene	0.67	1.16			1.61	0.52		0.12
trans-Cinerone							0.06	0.21
β-Ionone	0.88							
β-Ionone, methyl-		0.06						
β-Phenylethyl Isovalerate							0.03	

Compound	BAR	GRA	MAR	PHO	PIC	POR	SCH	UBA
FATTY ACID								
Dodecanoic acid		0.09						
Methyl-n-tridecanoate		0.37						
KETONE								
3-Penten-2-one							0.03	
Acetophenone	0.12			1.19			0.05	
Ethanone, 1-(2-methyl-2-cyclopenten-1-yl)-		0.02						
Sulcatone	0.52		0.37		0.32			
MONOTERPENE								
(R)-Lavandulyl	0.61							
(R)-Lavandulyl acetate					0.37			
1, 10-Limonenyl acetate				0.43				
1,2-Oxidolinalool					1.56			
1,3,6-Octatriene, 3,7-dimethyl-, (Z)-							14.60	9.58
3-Caranol							11.51	
3-Carene	1.03							
Azulene			0.41					
Carveol				24.22	14.17			
cis-Geraniol	2.65	0.98			1.97			
cis-Geranylacetone					1.56			
cis-Linalool	0.42						0.14	0.25
cis-p-Mentha-2,8-dien-1-ol								0.09
Citronellyl	0.80							
D-Limonene						2.79		
Estragole							23.6	20.71
Eugenol			0.76	9.32			3.45	
Geranylacetone	2.22	3.63		0.42	0.96	2.51		
Ipsenone	0.01							
Levomenthol			0.09					
Linalool					7.16			

Compound	BAR	GRA	MAR	PHO	PIC	POR	SCH	UBA
Methyl jasmonate						2.03		
Myrtenol								0.07
Photocitral B					0.01			
ρ-Menthatriene		0.07					0.25	
trans-3-Caren-2-ol								0.02
trans-Geranylacetone							7.84	0.10
trans-Linalool								0.06
trans-β-Ocimene		0.47						
α-Ocimene					10.52			
α-Pinene	0.39		6.23		1.77	1.26	0.03	
α-Pinene, (D)-	3.17							
α-Terpineol					7.33		0.89	
β-Citronellol	1.53	0.50			1.04			
β-Geraniolene					2.23		0.21	
β-Linalool		0.32					3.71	0.26
β-Myrcene					0.40			
β-Ocimene		73.48						
β-Phellandrene						0.01		
β-Pinene	0.77		0.81					
γ-Terpinene				0.43				0.20
SESQUITERPENE								
3,7-Cycloundecadien-1-ol, 1,5,5,8-tetramethyl-						2.39		
3-Methyl-apopinene							6.57	
4-*trans*,6-*cis*-Allocimene								0.50
7-epi-trans-sesquisabinene hydrate			2.41					
Cadina-1(10),4-diene	0.34		1.64					
Calacorene						0.74		
Caryophyllene	4.12	1.17	4.12	4.50	3.20		7.06	
cis-Caryophyllene					3.20			
Cosmene		0.28					0.10	1.12

Compound	BAR	GRA	MAR	PHO	PIC	POR	SCH	UBA
Cumene							0.31	
Cumene, 2,4,5-trimethyl-	0.36							
Cyclosativene							0.03	0.06
epi-Bicyclosesquiphellandrene							1.00	
Germacrene D		0.13						
t-Cadinol		0.18						
trans-Calamenene				0.12				
trans-α-Bergamotene							0.06	0.03
α-Acorenol	0.91							
α-Copaene						3.60	2.88	1.53
α-Cubebene							0.11	0.12
α-Farnesene	0.44		0.79				6.30	3.98
β-Bisabolene			1.52					
β-Bourbonene			0.52					
β-Cadinene							0.87	
β-Copaene	0.39		1.61		0.70	0.57	0.25	
β-Cubebene			0.53				0.37	0.28
β-Ylangene								0.60
γ-Cadinene							0.22	0.21
γ-Muurolene			0.43			1.38	0.16	0.15
δ-Elemene			0.55					
Specimen 1 (Total %)	**99.99995**	**99.9863**	**100.00**	**97.974**	**100.00**	**100.00**	**100.00**	**99.592**
Specimen 2 (Total %)	**99.97**	**100.00**	**-**	**99.99**	**99.95**	**-**	**100.00**	**-**
Specimen 1 (Unidentified VOCs %)	**0.00005**	**0.0137**	**0.00**	**2.026**	**0.00**	**0.00**	**0.00**	**0.408**
Specimen 2 (Unidentified VOCs %)	**0.03**	**0.00**	**-**	**0.01**	**0.05**	**-**	**0.00**	**-**

DISCUSSION

Our analyzes identified more diverse floral profiles than previously reported by Flach et al. (2004) (Tables 3-6). For *B. gracilis* we identified 63 compounds (against 34 of Flach et al. (2004), for *B. picta* 69 (against 9) and for *B. ubatubana,* 34 (against 16). The exception was *B. marginata* of which we identified 34 compounds against 35 from Flach et al. (2004). However, floral profiles in these four species had only from three to four floral VOCs in common between both studies (see Tables 3-6). Nonetheless, chemical categories of floral profiles were congruent between both studies for the same species.

We found the Tetrachloroethylene for *B. barbozae* (0.02%) and *B. picta* (0.07%). This volatile of amine group also was identified for other species of subtribe Maxillariinae (*Maxillariella variabilis* (Bateman ex Lindl.) M.A.Blanco & Carnevali, 1.36%) (Lipińska et al., 2021). Comparing our results with chemical analysis of *M. variabilis*, we verified similarities aromatic substances (phenols, aromatic carboxylic acids, mono- and sesquiterpenes). It is common in phenolic resins for lipophilic compounds to be mixed with terpenoids/phenolic compounds. The labellar secretion analysis of other species sampled by authors revealed that *Maxillariella sanguinea* (Rolfe) M.A.Blanco & Carnevali contains the monoterpene Limonene and the sesquiterpene Caryophyllene. Moreover, cinnamic acid was present in *M. sanguinea*, and Benzoic acid and its derivatives were found in all species (Langenheim, 2003).

We detected the volatile ethyl-2-butylnoate for *B. picta* (0.91%), not detected by Flach et al. (2004). This volatile was also found in the composition of sweet passion fruit (*Passiflora alata* Curtis) (Mamede et al., 2017). In this study, two genotypes (BGM004 and BGM163) and two SPME fibers were tested by authors. Forty-five volatile compounds were properly identified and quantified. Methyl butanoate, Ethyl (E)-2-butenoate, Ethylbutanoate, Ethyl (E)-2-butenoate, Methyl 2-hexenoate, and Ethyl-2-hexenoate were among major compounds. Similar result was obtained by Sampaio et al. (2015), where represented 21% of the total chromatogram area, especially Ethyl 2-hydroxyhexanoate, Ethyl trans-2-butenoate and Ethyl 2-methylbutanoate, and were responsible for the fruity/cashew-like aroma.

The volatile 6,19-Icosadiene detected only in our study was obtained in *B. barbozae* (1.39%). This alkadiene is associated with one of the major compounds active in the sex pheromone gland extract of *Eilema japonica* (Leech, 1888) lichen moth females (Arctiidae, Lithosiinae) (Fujii et al., 2010). According to the pheromone database,

alkadiene/alkenyl hydrocarbons are commonly used as sex pheromones in Arctiidae. However, it is necessary to obtain information on the sex pheromones of other lichen moths and which class of compounds is more common in this group of moths (Fujii et al., 2010).

Within Fatty acid group, we detected Dodecanoic acid (0.09%) and Methyl-*n*-tridecanoate (0.37%) in small fractions on the scent bouquet of *B. gracilis*. These volatiles were not observed by Flach et al. (2004). About these two volatiles, Laver e Fang (1986) verified them in Douglas-fir *Pseudotsuga menziesii* (Mirb.) Franco. Also released were several fatty acids: *n*-tridecanoic, *n*-hexadecanoic, *n*-heptadecanoic, *cis*-9-octadecenoic, *n*-nonadecanoic, *n*-eicosanoic, *n*-docosanoic, and *n*-tetracosanoic acid.

Exclusive volatiles in high percentages were detected in our and Flach et al. (2004) studies for *B. picta*: the alkenes E-14-Hexadecenal and E-15-Heptadecenal (37.48%; 9.32%) and the monoterpene Linalool (70.5%), respectively. The alkenes observed in our study were already verified by Satyal et al. (2015) for *Solanum xanthocarpum* Schrad. & J.C.Wendl. species and the authors concluded that have an optimal antibacterial and antioxidant activity. Karanja et al. (2021) showed through essential oil of *Solanum incanum* L. that the extract was a complex mixture of 15 bioactive compounds, such as: 2,4-di-tert-butylphenol, 1-Dodecene, E-14- Hexadecenal, E-15-Heptadecenal, *n*-Tetracosanol-1,9-Octadecenamid-(Z), among others. It also depicted that there is a greater potential of effective compounds from natural sources, which can contribute in control of plant pathogens. Yet, purified compounds have a higher potential of inhibiting microbe growth. The monoterpene Linalool detected in abundance by Flach et al. (2004) is a common floral volatile with related metabolites involved in the plant-pollinator interactions (Raguso, 2016). Studies revealed a complex interplay between pollinator attraction and plant defense mediated by Linalool and its derivatives, from the smallest (*Arabidopsis* and *Mitella*) to the largest *Datura stramonium* L. flowers (Chen et al., 2013). Fig wasps (Chen and Song, 2008), long tongued fungus gnats (Okamoto et al., 2015) and noctuid moths (Plepys et al., 2002; Dötterl et al., 2006) showed electrophysiological, neural and behavioral sensibility to different quantitative ratios of Linalool and Lilac compounds in floral bouquets. It is interesting to note that the diverse functions of Linalool, ranging from toxin to long-distance pollinator attractant are discussed in the context of floral volatile ecology and evolution (Raguso, 2016).

Only Flach et al. (2004) found Methyl-salicylate (MeSA) for *B. marginata* (0.6%). This volatile plant is a microbial signaling compound involved in systemic acquired resistance (SAR) and defense against pests and microbial pathogens, and antagonists. MeSA emitted by plants is also believed to trigger SAR in neighboring plant individuals, thus contributing to the resilience of the entire plant community (Singewar et al., 2021). MeSA also is produced in fungi and in higher plants and acts as a volatile signal between plants, between different fungi, and between plants and fungi. Furthermore, MeSA is one of the best-studied biogenic volatile organic compound (BVOC) linked to aphid *Myzus persicae* (Sulzer, 1776) infestation (Staudt et al., 2010). A study involving MeSA suggested that its role varies depending on plant and insect species (Yangang et al., 2020) and also be intricately related to insect-insect pheromone signaling (Xu and Turlings, 2018).

Flach et al. (2004) verified the presence of the aldehydes *n*-Nonanal and *n*-Decanal for *B. gracilis* and *B. marginata*, volatiles not identified in our study. None of the volatiles exceed the percentage of 5%. Similar result was observed by Jürgens et al. (2002) in *Silene vallesia* L. (Caryophyllaceae), in which common compounds among the fatty-acid derivatives were the aldehydes *n*-Octanal, *n*-Nonanal, and *n*-Decanal, with relative amount of each one never exceeding 5%. Another important point is about the vast majority of chemicals identified in *Silene* species that are common compounds of a wide array of scented *Brasiliorchis* species (Knudsen et al., 1993). These compounds include acyclic terpene alcohols (Linalool), aromatic alcohols (e.g. Benzyl alcohol, Phenylethyl alcohol), esters and small amounts of nitrogen-containing compounds. Also, terpenoids as geraniolic compounds, oxygenated sesquiterpenes, 1,8-Cineole and Linalool were identified (Knudsen et al., 1993).

Flowers follow rhythmic emission to emit scent volatiles floral mostly to conserve energy. It is mainly an evolutionary process where this temporal emission of scent volatiles possibly developed for interaction between the insect pollinators and flowers (Bera et al., 2017). In *Brasiliorchis* species have adapted to vegetative propagation, these cultivated sympatric species perhaps had weak intrinsic reproductive barrier. Thus, there was a need for prevention of interspecific pollination to maintain the species integrity. Hence, over the evolution the flowering time of these sympatric species might have shifted leading to floral isolation, the most important pre-zygotic barrier (Waelti et al., 2008). The enhanced atmospheric temperature during winter is different of summer, where an optimum atmospheric temperature should rise above 20°C for increased release

of floral scent. A temperature above 20°C is mostly observed in tropical winter between 12:00 p.m. and 4:00 p.m. We observed that even if the flower blooms in the early morning the maximum emission from *Brasiliorchis* species occurred at noon, which is because of the enhanced vaporization rate (Jakobsen and Olsen, 1994). The biosynthesis of floral volatiles within the flower tissue is a continuous process, where external factors, such as temperature, light and humidity might be responsible for slow release of the volatiles produced and stored in the flower tissues during the floral lifespan (Kondo et al., 2006). It is important to consider that such external factors may also have influenced the volatiles found in the present study.

An interesting phenomenon, which needs more evidence, is the suggested capacity of the flowers of specialist-pollinated plants to shift their emissions to attract a more generalist range of visitors when they remain unpollinated for a long time (Dudareva and Pichersky, 2000). A key-question that arises from the high diversity in emissions of floral volatiles (Majetic et al., 2009) is whether this plasticity is similar in plants with different levels of selective pressure acting on floral scent (such as specialist- and generalist-pollinated plants). Less variability in the profiles of floral scents is expected in plants experiencing higher selective pressures on floral scent as a reliable signal for pollinators. Some works have observed that deceptive species present a higher variability in traits associated with pollinator attraction, including floral scent, than rewarding species (e.g. Salzmann et al., 2007; Juillet and Scopece, 2010). Parachnowitsch et al. (2012) have provided an interesting work on phenotypic selection on floral scent. They demonstrate that selection towards higher floral emissions can be stronger than selection acting on other floral traits also related with pollinator attraction, such as flower colour and size. However, they did not make measurements to identify the agents of selection acting in their system and the specific importance of each one. New experiments should try to reveal the agents driving floral scent selection and the relative intensity of the selection pressures they exert.

The difference in the results obtained by us and by Flach et al. (2004) may be a consequence of the different methodology applied for VOC identification (Jelen et al., 2000). While Flach et al. (2004) used the VOCs elution procedure in hexane by solid phase microextraction analytical technique (SPME) after injected in GC-MS, we used dynamic headspace thermal desorption using helium as carrier gas in the GC-MS and TENAX-TA as the absorbent polymer. This difference is due to the fact that the technique used in this study with a thermal desorption system is more effective in preventing early

degradation of floral VOCs than elution of floral VOCs in a liquid substrate (hexane or similar). In this regard, the literature corroborates the efficiency of the system used here. Albertsson et al. (2006) showed that degradation of polymer polyamide 6,6 occurs earlier in the SPME method when compared to other methodologies. Polymer degradation may alter quantity and type of extracted VOCs (Thiébaut et al., 2007). Temperature used during sample analysis is also an important factor as it influences the quantity of VOCs adsorbed in the SPME polymer (Zhang and Pawliszyn, 1995). Also, collection time may influence VOC identification depending on the molecular size of compounds. The study of Kanavouras et al. (2005) with *Olea europaea* L. showed that an increase in collection time from 15 to 30 min resulted in an increase in the number of low molecular weight volatiles using the TENAX-TA method. Conversely, high molecular weight volatiles, such as Nonanal, Hexanol, Hexanal and Ethyl-2-methylbutirate, showed a difference in detection intensity. Kanavouras et al. (2005) also suggested that TENAX-TA has a greater efficiency in quantifying VOCs when compared to the SPME because the degradation of VOCs is smaller in the former.

Brasiliorchis species sampled in this study emitted particular floral bouquets, which clustered in distinct groups among species, suggesting that floral fragrance has a great interspecific variation. The capacity to evolve new pathways or modify existing pathways to synthesize blends of distinct floral VOCs is crucial for the evolutionary stability of pollination by a food-deceptive system (Schiestl and Schlüter, 2009). Flowers of rewardless Maxillariinae species emit strong fragrances, largely responsible for long-distance pollinator attraction. In observations of pollination biology, Singer and Cocucci (1999) indicate that the fragrances attract food-seeking stingless bees. In this study, the floral chemical composition for seven Maxillariinae species revealed a predominance of terpenoids (mono and sesquiterpenes), and aldehydes, aromatics and fatty acid derivatives among the minor categories. *B. gracilis* and *B. marginata* show similar fragrance compositions. The presence of 5-methyl, 3-heptanone could be associated with pheromonal activity. Pollinator behavior suggests that the bees may visit these orchids in search of food sources.

Pollinators are always modifying their behavior when recognizing deceptive flowers (Juillet et al., 2011), resulting in a small frequency of visitation in deceptive-pollinated flowers (Smithson and Macnair, 1997). Two hypotheses may explain high diversity of floral VOCs among morphologically very similar species such as observed for *Brasiliorchis* species. A first scenario is a result of negative frequency selection acting

on floral traits of food deceptive flowers. For example, the study of Gigord et al. (2001) showed pollinator preference for the rare-color morph of flowers of the orchid *Dactylorhiza sambucina* (L.) Soó. Alternatively, floral VOCs may differ as a result of no selection by pollinators. According to Smithson and Macnair (1997), selection is not likely to occur in food-deceptive species as pollinators do not learn to associate the flowers with resources, thus floral constancy will not be established. As there is no selection of flower traits by pollinators, the expectation would be that floral VOCs vary randomly (Juillet and Scopece, 2010). Salzmann et al. (2007) did not find a consistent correlation between particular scent profiles and relative fitness in food-deceptive orchids of genus *Orchis* L. Thus, odor differences between species may represent a neutral by-product of genetic drift after the speciation process rather than a result of pollinator-imposed selection (Huber et al., 2005). Deceptive systems can be based on visual or olfactory cues and usually involve only one or a few specialized pollinators (Newman et al., 2012). Pollinators often encounter empty flowers and this likely resulted in the lack of strong selection pressure vs. lack of reward (Gigord et al., 2001). Rewardless mimics or deceptive flowers are maintained by negative frequency dependence, where they are rare compared to rewarding model species. Persistence of such deceit pollination depends primarily on the pollinators' perceptual biases (Gigord et al., 2001; Jersáková et al., 2006). Furthermore, the evolution of deceptive pollination systems is a topic that has received little attention and will benefit from an understanding of the floral and pollinators phylogenies (Johnson et al., 2013), and also analysis of costs incurred by pollinators.

Lastly, environmental variables, such as soil, humidity and temperature, (Salzmann et al., 2007) can interfere in the biochemical pathways responsible for the synthesis of floral VOCs, resulting in the production of distinct compounds among distinct species inhabiting the same site (Coley et al., 1985) or among individuals of the same species (Majetic et al., 2009). For example, the study of Dobson et al. (1997) showed that the water stress in *Narcissus* species resulted in a different scent emission pattern and Nault (2003) verified that differences in temperature regimes between sites influenced vegetative emission of relative amounts of terpenoids in Douglas-fir *Pseudotsuga menziesii* (Mirb.) Franco. Also, different climatic factors within sites may cause differentiation in scent emission patterns in the populations in the same year, but may contribute even more to differentiation when samples of different years are compared among them (Salzmann et al., 2007). Furthermore, the drivers of global change can also act on at least two levels in the role of VOCs in plant-pollinator communication: 1)

affecting floral status and emissions, and 2) by affecting the properties of VOCs once they are released into the air. These two levels can synergistically or antagonistically affect the signals in floral scents for pollinators. These effects of global change need urgent research since a reduction in the capacity of pollinators to find flowers would have serious consequences in many plant communities and agriculture (Bartowska and Johnston, 2012).

TABLE 3. Comparison of relative abundance of floral volatile organic profiles of *B. gracilis* (Lodd.) R.B.Singer, S.Koehler & Carnevali obtained in this study and in Flach et al. (2004). Compounds in common are shown in bold type.

Compounds	**This study**	**Flach et al. (2004)**
ALCOHOL		
1-Dodecanol	1.14	
1-Dodecanol, 3,7,11-trimethyl-	1.04	
1-Hexanol, 2-ethyl-	12.44	
Cyclohexanol, 3,5-dimethoxy	0.10	
E-2-Hexadecacen-1-ol	0.22	
Hexanoic acid	0.13	
Hexanoic acid, 2-ethyl-	0.41	
Nonanoic acid	0.06	
ALDEHYDE		
(Z,Z)-3,6-nonadienal	0.37	
2-Decenal, (E)-	0.08	
Decanal	1.16	
Dodecanal	0.41	
Hexanal	0.38	
Lilial	0.16	
η-Decanal		4.9
η-Nonanal		3.0
Nonanal	0.51	
Octanal	0.40	
ALKANE		
Cyclododecane	0.03	
Dodecane, 2,6,10-trimethyl-	0.09	
Dodecane, 2,6,11-trimethyl-	0.17	
Dodecane, 2,7,10-trimethyl-	0.11	
Dodecane, 5,8-diethyl-	0.23	
Heptadecane	1.30	
Heptadecane, 2,6,10,15-tetramethyl-	0.03	

Compounds	This study	Flach et al. (2004)
Hexadecane	4.93	
Hexadecane, 2,6,10,14-tetramethyl-	0.48	
Nonane	0.26	
η-Tetradecane		0.7
Octadecane, 2-methyl-	0.03	
Octadecane, 3-ethyl-5-(2-ethylbutyl)-	0.27	
Octane	0.20	
Pentacosane, 13-phenyl-	0.20	
Pentadecane, 2,6,10,14-tetramethyl-	0.20	
Pentadecane, 2,6,10-trimethyl-	0.16	
Pentadecane, 2-methyl-	0.21	
Pentadecane, 3-methyl-	0.23	
Pentane, 2,3,4-trimethyl-	0.00	
Tetradecane	1.12	
Tetradecane, 2,6,10-trimethyl-	0.21	
trans-phyt-2-ene	0.15	
Tridecane	0.23	
Tridecane, 3-ethyl-	0.05	
Undecane, 2,9-dimethyl-	0.13	
Undecane, 3,7-dimethyl-	0.22	
ALKENE		
2,4,6,8-Tetramethyl-1-undecene	0.04	
2-Methyl-E-7-Hexadecene	0.41	
3-Octadecene, (E)-	0.44	
8-Heptadecene	0.34	
Cetene	0.72	
E-14-Hexadecenal	3.09	
AROMATIC		
Alloaromadendrene		1.0
Aromadendrene		0.1

Compounds	This study	Flach et al. (2004)
Butylated hydroxytoluene		2.6
Mesitylene		0.8
Naphtalene		3.0
ρ-*tert*-butylphenol	0.13	
Toluene	1.16	
β-Ionone, methyl-	0.06	
KETONE		
5-Methyl-3-heptanone		0.7
Acetophenone		0.3
Ethanone, 1-(2-methyl-2-cyclopenten-1-yl)-	0.02	
6-Methyl-5-hepten-2-one/Sulcatone		1.3
MONOTERPENE		
cis-β-Ocimene		0.2
cis-Geraniol	0.98	
Geranylacetone	**3.63**	**0.9**
Limonene		0.2
Linalool		2.8
ρ-Cymene		0.2
ρ-Menthatriene	0.07	
trans-Linalool		0.3
***trans*-β-Ocimene**	**0.47**	**30.3**
β-Citronellol	0.50	
β-Linalool	0.32	
β-Ocimene	73.49	
SESQUITERPENE		
Caryophyllene	1.17	
cis-α-Bergamotene		0.3
Cosmene	0.28	
Germacrene D	**0.13**	**0.2**

Compounds	**This study**	**Flach et al. (2004)**
t-Cadinol	0.18	
trans-Caryophyllene		0.1
α-Calacorene		0.4
α-Copaene		5.7
α-Cubebene		0.6
α-Gurjunene		0.1
α-Humulene		0.1
α-Muurolene		1.7
α-Selinene		0.1
α-Ylangene		0.2
β-Elemene		0.2
β-Gurjunene		0.1
γ-Muurolene		0.6
δ-Cadinene		9.7
δ-Elemene		0.1
Total of compounds	**63**	**34**

TABLE 4. Comparison of relative abundance of floral volatile organic profiles of *B. marginata* (Lindl.) R.B.Singer, S.Koehler & Carnevali obtained in this study and in Flach et al. (2004). Compounds in common are shown in bold type.

Compounds	**This study**	**Flach et al. (2004)**
ALCOHOL		
1-Octanol	27.26	
Octanoic acid	36.43	
ALDEHYDE		
2-Decenal, (E)-	0.06	
Butanal	0.07	
Decanal	1.40	
Heptanal	0.23	
η-Decanal		4.4
η-Nonanal		1.9
Nonanal	2.43	
Octanal	0.49	
ALKANE		
Decane, 2,3,5-trimethyl-	0.26	
Dodecane		0.8
Dodecane, 2,7,10-trimethyl-	0.47	
Heptadecane	0.53	
Hexadecane	2.99	
η-Tetradecane		0.9
Tetradecane	0.72	
Tridecane	0.54	
Tridecane, 2-methyl-	0.89	
AROMATIC		
Alloaromadendrene		2.0
Aromadendrene		0.1

Compounds	This study	Flach et al. (2004)
Benzaldehyde	0.79	
Benzoic Acid	0.31	
Butylated hydroxytoluene		17.7
Mesitylene		0.8
Naphtalene		2.9
Phenylethyl Alcohol	1.06	
ESTER		
Methyl salicylate		0.6
KETONE		
Acetophenone		0.3
Sulcatone	**0.37**	**2.6**
5-Methyl-3-heptanone		0.5
MONOTERPENE		
Azulene	0.41	
Eugenol	0.76	
Geranylacetone		1.5
Levomenthol	0.09	
Limonene		9.0
Linalool		0.2
trans-Linalool		0.5
trans-β-Ocimene		1.3
α-Pinene	**6.23**	**0.4**
β-Pinene	0.81	
SESQUITERPENE		
1-epi-Cubenol		0.1
7-epi-trans-sesquisabinene hydrate	2.41	
Cadina-1(10),4-diene	1.64	

Compounds	**This study**	**Flach et al. (2004)**
Caryophyllene	4.12	
cis-α-Bergamotene		0.4
Germacrene D		1.0
Khusimene		0.1
trans-Caryophyllene		0.4
ar-Curcumene		1.9
α-Calacorene		0.6
α-Copaene		9.6
(E, E) α-Farnesene		0.6
α-Farnesene	0.79	
α-Muurolene		2.0
α-Selinene		0.3
β-Bisabolene	1.52	
β-Bourbonene	0.52	
β-Copaene	1.61	
β-Cubebene	0.53	
β-Elemene		0.6
β-Gurjunene		0.2
γ-Muurolene	**0.43**	**1.0**
δ-Cadinene		11.6
δ-Elemene	**0.55**	**0.1**
Total of compounds	**34**	**35**

TABLE 5. Comparison of relative abundance of floral volatile organic profiles of *B. picta* (Hook.) R.B.Singer, S.Koehler & Carnevali obtained in this study and in Flach et al. (2004). Compounds in common are shown in bold type.

Compounds	**This study**	**Flach et al. (2004)**
ALCOHOL		
1-Dodecanol, 3,7,11-trimethyl-	0.12	
1-Hexanol, 2-ethyl-	3.58	
1-Tetradecanol	1.15	
ALDEHYDE		
1-Hexadecanol	0.73	
1-Hexadecanol, 2-methyl-	1.86	
2-Undecenal	4.09	
3-Methyl-3-cyclohexen-1-one	0.19	
Dodecanal	1.94	
Heptanal	0.25	
η-Decanal		2.6
Undecanal	1.93	
Z-10-Pentadecen-1-ol	0.41	
ALKANE		
2-Hexadecanol	0.69	
3-Methyleicosane	1.86	
Decane, 2,3,5-trimethyl-	0.16	
Dodecane	3.61	
Dodecane, 2,6,10-trimethyl-	0.08	
Dodecane, 2,6,11-trimethyl-	0.13	
Heptadecane	5.26	
Heptadecane, 2,6,10,14-tetramethyl-	2.64	
Heptane, 5-ethyl-2-methyl-	0.11	
Hexadecane	3.76	
Hexadecane, 2,6,10,14-tetramethyl-	0.31	

Compounds	This study	Flach et al. (2004)
Nonane, 2,6-dimethyl	0.03	
Pentadecane, 2-methyl-	0.54	
Pentadecane, 3-methyl-	0.35	
tert-Hexadecanethiol	0.55	
Tetradecane	8.64	
trans-phyt-2-ene	1.44	
Tridecane	1.68	
Tridecane, 2-methyl-	0.24	
Undecane	0.18	
Undecane, 2,6-dimethyl-	0.36	
Undecane, 4,7-dimethyl-	0.15	
Undecane, 4,8-dimethyl-	0.58	
Undecane, 5,7-dimethyl-	0.29	
ALKENE		
11-Tricosene	0.37	
1-Docosene	2.17	
1-Pentadecene	0.57	
5-Eicosene, (E)-	2.89	
8-Heptadecene	2.40	
Cetene	3.87	
E-14-Hexadecenal	37.48	
E-15-Heptadecenal	9.32	
ALKYNE		
Ethyl 2-butynoate	0.91	
AMINE		
Tetrachloroethylene	0.07	

Compounds	This study	Flach et al. (2004)
AROMATIC		
Benzeneacetaldehyde	5.42	
Mesitylene	0.13	
ρ-Anisaldehyde	8.71	
Phenylethyl alcohol	2.93	
ρ-tert-butylphenol	0.18	
Toluene	1.61	
KETONE		
Dihydro-β-ionone		9.6
6-Methyl-5-hepten-2-one/Sulcatone	**0.32**	**1.1**
MONOTERPENE		
(R)-Lavandulyl acetate	0.37	
1,2-Oxidolinalool	1.56	
Carveol	14.17	
cis-Geraniol	1.97	
cis-Geranylacetone	1.56	
Geranylacetone	0.96	
Linalool	**7.16**	**70.5**
Photocitral B	0.01	
α-Ocimene	10.52	
α-Pinene	**1.77**	**5.8**
α-Terpineol	7.33	
β-Citronellol	1.04	
β-Geraniolene	2.23	
β-Myrcene	0.40	
SESQUITERPENE		
Cadalene		1.4

Compounds	**This study**	**Flach et al. (2004)**
Caryophyllene	3.20	
cis-α-Bergamotene		1.1
cis-Caryophyllene	3.20	
α-Ylangene		2.0
β-Copaene	0.70	
δ-Cadinene		1.1
Total of compounds	**69**	**9**

TABLE 6. Comparison of relative abundance of floral volatile organic profiles of *B. ubatubana* (Hoehne) R.B.Singer, S.Koehler & Carnevali obtained in this study and in Flach et al. (2004). Compounds in common are shown in bold type.

Compounds	**This study**	**Flach et al. (2004)**
ALCOHOL		
Hexanoic acid	10.58	
ALDEHYDE		
4-Nonenal, (E)-	0.09	
cis-3-Hexenol	0.25	
Dodecanal	3.39	
Hexanal	0.07	
η-Decanal		0.3
η-Octanal		0.1
ALKANE		
Dodecane, 2,7,10-trimethyl-	0.02	
AROMATIC		
Aromadendrene	0.17	
Benzaldehyde		2.1
Benzyl alcohol		0.3
Cinnamaldehyde, (E)-	4.78	
Indole-2,3-dione	0.75	
ρ-Anisaldehyde	22.90	
Phenylacetaldehyde		47.5
Phenylethyl alcohol	**16.35**	**35.7**
Toluene	0.12	
trans-Cinerone	0.21	
β-Ionone, methyl-		

KETONE		
6-Methyl-5-hepten-2-one/Sulcatone		0.1
MONOTERPENE		
1,3,6-Octatriene, 3,7-dimethyl-, (Z)-	9.58	
1,8-Cyneol/Eucalyptol		0.1
***cis*-Linalool**	**0.25**	**0.1**
cis-ρ-Mentha-2,8-dien-1-ol	0.09	
Estragole	20.71	
Limonene		0.1
Linalool		3.8
Myrtenol	0.07	
trans-3-Caren-2-ol	0.02	
trans-Geranylacetone	0.10	
trans-Linalool	0.06	
trans-β-Ocimene		3.7
α-Pinene		2.4
α-Terpineol		1.4
β-Linalool	0.26	
γ-Terpinene	0.20	
SESQUITERPENE		
4-*trans*, 6-*cis*-Allocimene	0.50	
Cosmene	1.12	
Cyclosativene	0.06	
trans-α-Bergamotene	0.03	
α-Cadinene		0.2
α-Copaene	**1.53**	**0.2**
α-Cubebene	0.12	
α-Farnesene	3.98	
β-Cubebene	0.28	

β-Ylangene	0.60	
γ-Cadinene	0.21	
γ-Muurolene	0.15	
Total of compounds	**34**	**16**

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CONSIDERAÇÕES FINAIS E PERSPECTIVAS

O presente estudo aborda o sistema reprodutivo e a química de fragrâncias florais de orquídeas do gênero *Brasiliorchis*, com o intuito de contribuir para o entendimento de processos biológicos atuantes em orquídeas Neotropicais.

A partir dos resultados de sistema reprodutivo e ecologia química, podemos concluir que as espécies estudadas dependem da polinização para o desenvolvimento de frutos e sementes. Outro ponto importante é que as espécies estudadas não possuem sistema de reprodução assexuada, sendo necessária a presença dos polinizadores para transferência polínica. Nós também elucidamos que *Brasiliorchis picta* apresenta um sistema de autoincompatibilidade gametofítica, uma vez que todos os tubos polínicos são degradados no canal estilar entre os 15-16 dias após as flores serem autopolinizadas. As espécies *B. phoenicanthera* e *B. porphyrostele* apresentaram total aborto dos frutos ou apenas um único fruto desenvolvido na autopolinização, respectivamente. Estes dados sugerem que o sistema de autoincompatibilidade ocorre dentro do gênero *Brasiliorchis*, e também é interessante analisar a possibilidade de sistemas de autoincompatibilidade operando em outros gêneros da subtribo Maxillariinae. Os sistemas de reprodução são afetados por diversos fatores externos às plantas, como vetores e comportamento de polinização, e, dentro das plantas, por fatores fenológicos e alocação de recursos dentro dos frutos. Estes fatores podem gerar variações na taxa de cruzamento e correlação de paternidade em frutos de diferentes espécimes, e entre espécimes intra e interpopulações. O conhecimento da variação nos sistemas de reprodução em populações de uma espécie é de fundamental importância para o desenho de estratégias de conservação e melhoramento genético, pois permite a aplicação de métodos de amostragem mais eficazes e o uso de modelos matemáticos mais realistas no estudo da herança quantitativa de caracteres de interesse econômico. Mais estudos considerando clados sem estudos de delimitação de espécies são necessários para testar a hipótese de sistemas de autoincompatibilidade como condutores de especiação na subtribo Maxillariinae.

O presente estudo também contribui com o avanço do conhecimento sobre a composição química de fragrâncias florais no gênero *Brasiliorchis*. As oito espécies estudadas apresentaram fragrâncias florais de composição muito distintas entre si. A grande variação encontrada pode influenciar a atração dos polinizadores, pois provavelmente essas são orquídeas de engodo, que evoluíram para a produção de distintos bouquets de fragrâncias florais, retardando a cognição dos polinizadores e aumentando as chances de reprodução. Uma vez que a composição de fragrâncias entre diferentes espécies amostradas em nosso estudo e no estudo já disponível de

Flach et al. (2004) é muito distinta, seja pelas diferentes metodologias de coletas empregadas em ambos os estudos, ou pela plasticidade dos COVs florais, pode-se ressaltar a imprescindibilidade de estudos intraespecíficos (i.e. entre diferentes indivíduos da mesma espécie) para a compreensão da real extensão da variação em COVs florais de orquídeas *Brasiliorchis*. Além disso, os impulsionadores da mudança global também podem atuar em pelo menos dois níveis no papel dos COVs na comunicação planta-polinizador e consequentemente na biologia reprodutiva das espécies: 1) afetando o status floral e as emissões de COVs, e 2) afetando as propriedades dos COVs quando são liberados no ar/ambiente. Estes dois níveis podem afetar sinergicamente, adicionalmente ou antagonicamente os sinais em fragrâncias florais para os polinizadores. Estes efeitos da mudança global precisam de pesquisas urgentes, pois a redução na capacidade dos polinizadores de localizar flores acarretaria sérias consequências nas comunidades vegetais e na agricultura.

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APÊNDICE I. RELATÓRIO DO SISGEN

Ministério do Meio Ambiente
CONSELHO DE GESTÃO DO PATRIMÔNIO GENÉTICO

SISTEMA NACIONAL DE GESTÃO DO PATRIMÔNIO GENÉTICO E DO CONHECIMENTO TRADICIONAL ASSOCIADO

Comprovante de Cadastro de Acesso

Cadastro nº A144711

A atividade de acesso ao Patrimônio Genético, nos termos abaixo resumida, foi cadastrada no SisGen, em atendimento ao previsto na Lei nº 13.123/2015 e seus regulamentos.

Número do cadastro:	**A144711**
Usuário:	**Nicolas Alberto Polizelli Ricci**
CPF/CNPJ:	**401.670.298-43**
Objeto do Acesso:	**Patrimônio Genético**
Finalidade do Acesso:	**Pesquisa**

Espécie
Brasiliorchis picta
Brasiliorchis barbosae
Brasiliorchis chrysantha
Brasiliorchis gracilis
Brasiliorchis marginata
Brasiliorchis phoenicanthera
Brasiliorchis porphyrostele
Brasiliorchis ubatubana
Brasiliorchis schunkeana
Brasiliorchis consanguinea
Trigonidium obtusum

Título da Atividade:	**Chemical ecology and reproductive systems in deceptive Maxillariinae orchids**

Equipe

Nicolas Alberto Polizelli Ricci	**UNICAMP**
Samantha Koehler	**UNICAMP**

Data do Cadastro: **08/08/2019 21:03:21**

Situação do Cadastro: **Concluído**

Conselho de Gestão do Patrimônio Genético
Situação cadastral conforme consulta ao SisGen em **11:28** de **25/02/2022**.

SISTEMA NACIONAL DE GESTÃO DO PATRIMÔNIO GENÉTICO E DO CONHECIMENTO TRADICIONAL ASSOCIADO - **SISGEN**

APÊNDICE II. DECLARAÇÃO DE BIOÉTICA E BIOSSEGURANÇA

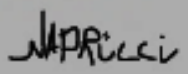
COORDENADORIA DE PÓS-GRADUAÇÃO – INSTITUTO DE BIOLOGIA
Universidade Estadual de Campinas
Fone (19)3521-6378, email: cpgib@unicamp.br

DECLARAÇÃO

Em observância ao §5° do Artigo 1° da Informação CCPG-UNICAMP/001/15, referente à Bioética e Biossegurança, declaro que o conteúdo de minha Tese de Doutorado, intitulada **"REPRODUCTIVE BIOLOGY AND FLORAL FRAGRANCES DIVERSITY OF *Brasiliorchis* ORCHIDS"**, 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 à Biossegurança.

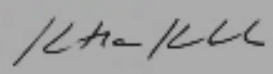
Nome do aluno: Nicolas Alberto Polizelli Ricci

Assinatura:



Nome da orientadora: Samantha Koehler

Assinatura:



Data: 02 de março de 2022