UNIVERSIDA DE FEDERAL DO RIO GRANDE DO SUL
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PROGRAMA DE PÓS-GRADUAÇÃO EM BOTÂNICA

DESVENDANDO OS SEGREDOS ÍNTIMOS DE UMA PETUNIA SECRETA

TESE DE DOUTORADO

DANIELE MUNARETO RODRIGUES

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FLUXO GÊNICO E BIOLOGIA REPRODUTIVA DE *PETUNIA SECRETA* STEHMANN & SEMIR

Daniele Munareto Rodrigues

Orientadora: Loreta Brandão de Freitas

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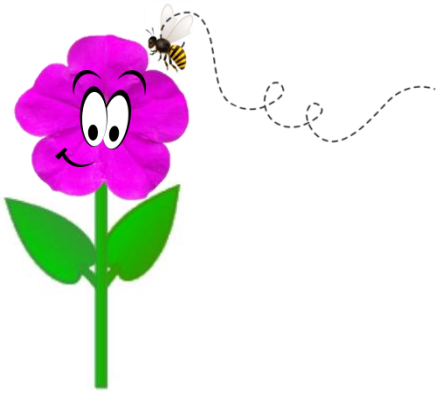

“...que a gente saiba FLORIR, onde a vida nos plantar.”

Rosi Coelho

RESUMO

Petunia secreta é uma espécie rara e endêmica da região chamada Pedra do Segredo, localizada em Caçapava do Sul, Rio Grande do Sul, Brasil. Filogeneticamente forma um clado dentro do gênero *Petunia* com outras duas espécies, *P. axillaris* e *P. exserta*, e juntas contam uma história de diversificação mediada pela polinização. Enquanto 12 espécies do gênero, incluindo *P. secreta*, são polinizadas por abelhas, *P. axillaris* é polinizada por mariposas e *P. exserta* por beija-flores. Muitos estudos foram realizados para entender a biologia reprodutiva e polinização envolvendo *P. axillaris* e *P. exserta*, mas pouco se sabe a respeito de *P. secreta*. O objetivo do presente trabalho foi gerar dados ecológicos e moleculares para a espécie *P. secreta*, através da investigação da polinização, biologia reprodutiva e fluxo gênico, contribuindo assim para a compreensão da história evolutiva dentro do clado "tubo longo". A investigação consistiu de observações na natureza, registro do polinizador, coleta de sementes, experimentos em casa de vegetação e caracterização genética, que foram avaliados com métodos de análise populacional e fluxo gênico. Os resultados obtidos revelaram que o polinizador efetivo de *P. secreta* é uma espécie de abelha do gênero *Pseudagapostemon,* que coleta exclusivamente pólen. Análise morfológica mostrou que *P. secreta* possui corola que reflete luz ultravioleta, característica essa que provavelmente selecionou a abelha como polinizador. Os experimentos em casa de vegetação comprovaram que *P. secreta* não possui autopolinização espontânea, confirmando a dependência de seu polinizador para sua reprodução. Esses experimentos também mostraram que *P. secreta* é autocompatível e a viabilidade das sementes independe do modo de fecundação. Os resultados de diversidade genética mostraram que *P. secreta* tem baixo índice de heterozigosidade e as análises de paternidade da progênie demonstraram que *P. secreta,* apesar de possuir um sistema misto de cruzamento, reproduz-se essencialmente por autofecundação, que sugerimos ser promovida pelo comportamento de coleta do polinizador. A morfologia da flor, a posição de depósito do pólen após a coleta e o comportamento da abelha para coletar todo o conteúdo polínico disponível, associados a um provável mecanismo de descarregar o pólen no ninho a cada visita floral, favorecem a autofertilização, contribuindo muito pouco para a polinização cruzada. As análises também mostraram que os cruzamentos foram endogâmicos, possivelmente devidos às curtas distâncias percorridas pelas abelhas e pela composição das populações de *P. secreta*, uma vez que o sistema de dispersão das sementes é por autocoria o

que favorece que indivíduos aparentados permaneçam próximos à planta mãe. As análises de paternidade também demostraram que o fluxo gênico entre as populações é nulo. Esses resultados sugerem a hipótese de que a formação de um banco de sementes possa manter a variabilidade genética ao longo do tempo, misturando indivíduos de diferentes gerações em uma mesma estação, e explicar os resultados encontrados que mostram *P. secreta* como uma espécie com alta variabilidade apesar de sua raridade.

Palavras-chave: Solanaceae, *Petunia*, polinização, abelha, sistema reprodutivo, fluxo gênico.

ABSTRACT

Petunia secreta is a rare and endemic species from a region called Pedra do Segredo, in Caçapava do Sul, Rio Grande do Sul, Brazil. Phylogenetically it belongs to a clade in the *Petunia* genus as sister of two other species, *P. axillaris* and *P. exserta*, and together they tell a history on diversification mediated by pollinator-adaptation. Whereas 12 *Petunia* species, including *P. secreta*, are bee-pollinated, *P. axillaris* presents moth-pollination and *P exserta* is pollinated by hummingbirds. Many studies have been carried out to understand the reproductive biology and pollination involving *P. axillaris* and *P. exserta*, but little is known about *P. secreta*. The objective of the present work was to generate ecological and molecular data to *P. secreta*, through the investigation of pollination, reproductive biology, and gene flow, thus contributing to the knowledge of the evolutionary history within the "long tube" clade. The research consisted of observations in nature, pollinator record, seed collection, greenhouse experiments, and genetic characterization that were evaluated with methodologies from population analysis and gene flow. The results obtained showed that the effective *P. secreta* pollinator is a bee species of the genus *Pseudagapostemon*, which exclusively collects pollen. Morphological analysis demonstrated that *P. secreta* has a corolla that reflects ultraviolet light, a characteristic that probably selected the bee as a pollinator. The experiments in the greenhouse proved that *P. secreta* does not have spontaneous self-pollination, confirming the dependence on this pollinator for its maintenance. These experiments also showed that *P. secreta* is self-compatible and the viability of the seeds is independent from the mode of fertilization. The results of genetic diversity showed that *P. secreta* have a low index of heterozygosity. The paternity analysis of the progeny showed that *P. secreta* despite having a mixed crossing system is essentially self-pollinated, which we suggest to be promoted by pollinator collection behavior, since it was observed that the bee probably discharges the pollen into the nest at each floral visit, thus contributing very little to cross-pollination. The analyses also showed that the crosses were performed by endogamy, possibly because the system of seed dispersal that is by *autochory*. The paternity analyses also showed that there is no gene flow among the populations. These results suggest the hypothesis that the seed bank could preserve the genetic variability over time and may explain recently finds that show *P. secreta* is a species with high genetic variability despite its rarity.

Key words: Solanaceae, *Petunia*, pollination, bee, mating system, gene flow.

SUMÁRIO

CAPÍTULO I

INTRODUÇÃO

1. Gênero *Petunia* Juss.

Petunia Juss. é um gênero endêmico da América do Sul, pertencente à família Solanaceae L. (APG III, 2009). O gênero foi inicialmente descrito por Jussieu em 1803 e nele foram incluídas cerca de 30 espécies com distribuição geográfica limitada ao sul da América do Sul, principalmente os estados do Paraná, Santa Catarina e Rio Grande o Sul, Brasil, o território do Uruguai e algumas províncias da Argentina (Stehmann, 1999; Stehmann & Semir, 2005). Através de estudos realizados por Wijsman (1982, 1983), foi verificado que o gênero *Petunia* como inicialmente delimitado era, na verdade, composto por dois grupos cromossômicos, um com espécies apresentando 2n=14 e outro com espécies 2n=18. Além da diferença no número, foram verificadas diferenças em morfologia e tamanho cromossômico. Além disto, experimentos de cruzamento entre espécies dos dois diferentes grupos não produziam híbridos viáveis, enquanto que ao cruzar espécies com um mesmo número cromossômico obtinham-se sementes férteis, independentemente da combinação (Wijsman, 1983). Desta forma, Wijsmann & Jong (1985) propuseram a separação destes grupos em dois diferentes gêneros. Inicialmente, seriam mantidas no gênero *Petunia* as espécies relacionadas à *Petunia parviflora* Juss. que apresentavam 2n=18, enquanto as espécies com 2n=14 seriam transferidas para o gênero *Stimoryne* Rafin. Porém, como o nome *Petunia* já estava associado à *Petunia X hybrida* (Hook.) Vilm., uma espécie de interesse ornamental popularmente conhecida como petúnia-de-jardim, Wijnands *et al.* (1986) propuseram a conservação de *Petunia nyctaginiflora* Juss. como o tipo nomenclatural para as espécies com 2n=14, ao mesmo tempo que as espécies com 2n=18 foram validadas para o gênero *Calibrachoa* La Llave & Lexarza (Wijnands *et al.*, 1986; Brummitt, 1989; Stehmann & Semir, 1997). Essa separação em dois gêneros distintos foi posteriormente confirmada em diversos estudos moleculares (Ando *et al.*, 2005; Kulcheski *et al.*, 2006; Olmstead *et al.*, 2008; Särkinen *et al.*, 2013).

Atualmente são descritas 14 espécies para o gênero, distribuídas nas regiões subtropicais e temperadas da América do Sul: *P. altiplana* T.Ando & Hashim, *P. axillaris* Britton, Sterns & Poggenb. (com três subespécies), *P. bajeensis* T.Ando & Hashim, *P. bonjardinensis* T.Ando & Hashim, *P. exserta* Stehmann, *P. inflata* R.E.Fr., *P. integrifolia* (Hook.) Schinz & Thell. (com duas subespécies), *P. interior* T.Ando & Hashim, *P. mantiqueirensis* T.Ando & Hashim, *P. occidentalis* R.E.Fr., *P. reitzii* L.B.Sm. & Downs, *P. saxicola* L.B.Sm. & Downs, *P. scheideana* L.B.Sm. & Downs, *P. secreta* Stehmann & Semir.

(Stehmann *et al.*, 2009). Destas espécies, apenas *P. occidentalis* não ocorre no Brasil, apresentando uma distribuição geográfica disjunta do restante do grupo, restrita às montanhas da região sub-Andina, no noroeste da Argentina e sul da Bolívia (Stehmann *et al.*, 2009). De acordo com a diversidade morfológica e aspectos ecológicos, são propostos dois centros de diversidade para o gênero: Campos de altitude da borda oriental do Planalto Catarinense e afloramentos rochosos areníticos (conglomerado) da Serra do Sudeste gaúcha (Stehmann *et al.*, 2009).

Filogenias do gênero *Petunia* construídas a partir de dados do DNA de cloroplasto separaram o gênero em dois clados principais, relacionados com a altitude de ocorrência das espécies ao longo da distribuição geográfica. Um clado formado por espécies que ocorrem entre 0 e 500 metros acima do nível do mar e que foi chamado de terras baixas, e outro clado formado por espécies que ocorrem entre 500 e 900 metros, chamado terras altas (Ando *et al.*, 2005; Kulscheski *et al.*, 2006). A filogenia de Chen *et al.* (2007) baseada na sequência do gene nuclear *Hf1* (Flavonoide-3',5'-hydroxilase) recuperou dois clados principais, mas com composição diversa da obtida por marcadores plastidiais e não associados à elevação onde as espécies ocorrem. Com exceção do posicionamento de *P. occidentalis*, as demais espécies foram agrupadas em função do comprimento do tubo da corola (Figura 1). Filogenia com a mesma topologia da apresentada por Chen *et al.* (2007) foi obtida para sequências de elementos de transposição *Tnt-1* (Kriedt *et al.*, 2014).

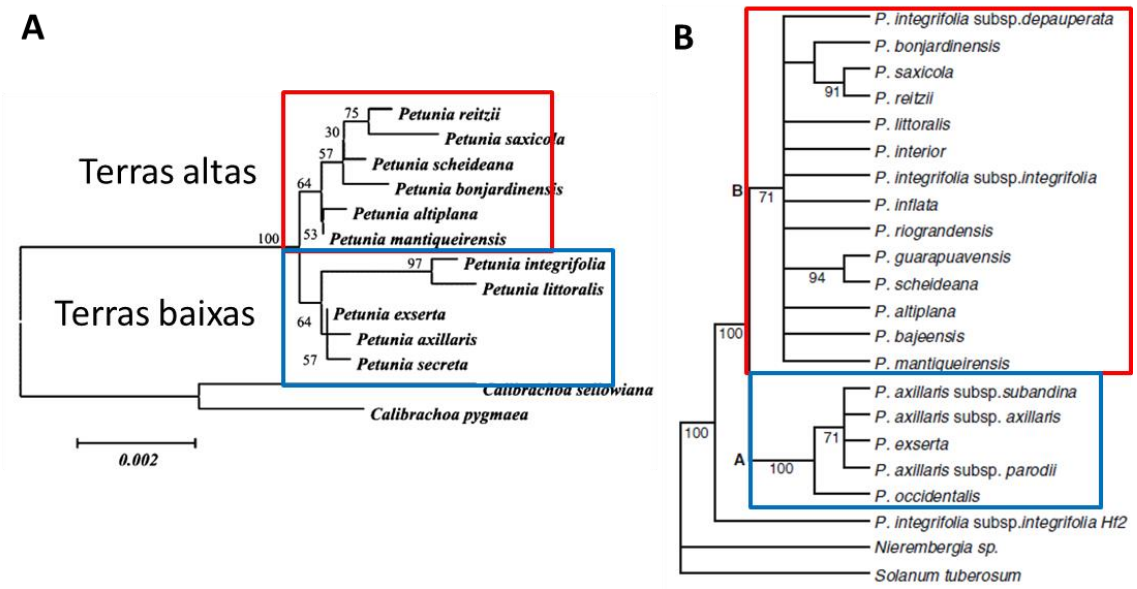

Figura 1. Topologias obtidas a partir de reconstruções filogenéticas baseadas no cpDNA (A) (Modificado de Kulscheski *et al.* (2006)) e nuDNA(B) (Modificado de Chen *et al.* (2007)).

Reck-Kortmann *et al.* (2014) realizaram a mais completa análise filogenética para o gênero até o momento, incluindo múltiplos locos plastidiais e nucleares e todos os diferentes *taxa* atualmente reconhecidos em *Petunia*. À semelhança do que foi observado nas filogenias de genes nucleares, os clados obtidos corresponderam a espécies que compartilham o comprimento do tubo da corola, de forma que elas podem ser divididas em espécies de "tubo curto" e espécies de "tubo longo" (Figura 2). O clado denominado "tubo curto" compreende 10 espécies, as quais apresentam flores de coloração púrpura, pólen azul, pouco néctar e sem aromas (Stehmann *et al.*, 2009). Enquanto no clado "tubo longo" estão reunidas três espécies que apresentam pólen amarelo: *P. axillaris* tem flores brancas, muito néctar e aroma crepuscular (Stuurman *et al.*, 2004; Galliot *et al.*, 2006); *P. exserta* tem flores vermelhas, estruturas reprodutivas exsertas, muito néctar e não possui aromas (Stehmann *et al.*, 2009); e *P. secreta* que possui flores magenta, pouco néctar e sem aromas (dados deste trabalho). Associada a estas três espécies, ainda neste clado, é encontrada *P. occidentalis* que compartilha as características morfológicas típicas das espécies do clado de "tubo curto".

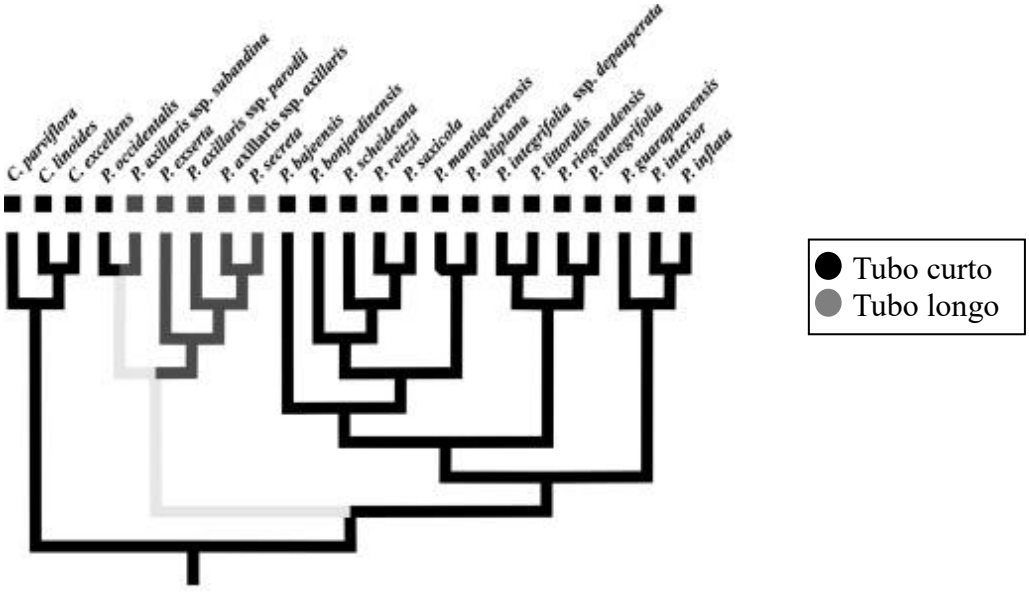

Figura 2. Clados tubo curto e tubo longo adaptado de Reck-Kortmann *et al.* (2014).

2. Polinização em *Petunia*

Petunia é um grupo referência para estudos de especiação mediada por polinização (Gübitz *et al.*, 2009; Vandenbussche *et al.*, 2016). Todas as espécies de "tubo curto" são

polinizadas por abelhas (Wittmann *et al.*, 1990; Ando *et al.*, 2001), enquanto que nas espécies de "tubo longo" encontramos três síndrome de polinização: *P. axillaris* polinizadas por mariposas (Ando *et al.*, 2001), *P. exserta* polinizada por beija-flores (Stehmann, 1987) e *P. secreta* polinizada por abelhas (Stehmann & Semir, 2005).

A transição de um tipo de polinizador para outro promove o isolamento reprodutivo e consequentemente a diversificação das espécies (Kay & Sargent, 2009). Essa transição requer alterações de traços florais como cor, aroma, composição e quantidade de néctar e morfologia floral (Armbruster & Muchhala, 2009). Em *Petunia,* diversos estudos vêm tentando elucidar como as características surgiram e foram selecionadas pelos diferentes polinizadores. As inferências filogenéticas mostraram que a polinização por abelhas nas espécies de *Petunia* representa o estado ancestral no gênero (Reck-Kortmann *et al.*, 2014). Ainda não se sabe exatamente que características isolaram as espécies primeiro, mas dados moleculares (Quattrocchio *et al.*, 1999; Ando *et al.*, 2005; Kulcheski *et al*., 2006; Hoballah *et al.*, 2007; Sheehan *et al.*, 2016) sugerem que a polinização por mariposas em *P. axillaris* evoluiu de polinização por abelha no ancestral comum de *P. axillaris* e das demais espécies de "tubo longo" e que a polinização por beija-flores em *P. exserta* evoluiu a partir da polinização por mariposa.

Vários estudos têm sido conduzidos sobre traços florais atraindo polinizadores em espécies de *Petunia*. Esses estudos incluíram análises da composição química de aroma e néctar (Sheehan *et al.*, 2012; Gleiser *et al.*, 2014), caracterização molecular de genes (Quattrocchio *et al.*, 1999; Hermann & Kuhlemeier, 2011; Hermann *et al*., 2015) e uma série de comparações sobre o comportamento e preferências dos polinizadores (Hoballah *et al.*, 2007; Venail *et al.*, 2010; Dell'Olivo & Kuhlemeier, 2013).

No caso do gênero *Petunia*, a reflexão e absorção de luz ultravioleta têm sido considerada a característica que pode ter influenciado diretamente a mudança de polinizador e, consequentemente, contribuído para a diversificação das espécies do clado tubo longo. As antocianinas e flavonóis são dois pigmentos vegetais responsáveis pela coloração que varia de vermelho vivo à violeta e de branco a amarelo claro, respectivamente (Quattrocchio *et al.*, 1999). As antocianinas são pigmentos que refletem luz ultravioleta e os flavonóis absorvem luz ultravioleta. As antocianinas e flavonóis competem pelos mesmos precursores nas vias metabólicas, isso leva a flores com cores diferentes dependendo da quantidade e do tipo de pigmento que está sendo prioritariamente expresso (Winkel-Shirley, 2001). Insetos polinizadores como as abelhas são atraídos pela coloração e o cheiro das flores (Grotewold,

2016). Durante o forrageio, a primeira sinalização para as abelhas encontrarem as flores é a visual. As abelhas possuem um sensor que detecta comprimento de onda ultravioleta, encontrando flores que refletem os raios ultravioleta provenientes do sol. Já insetos como as mariposas tem preferência por flores que absorvem UV (White *et al.*, 1994). No clado de tubo curto, as flores refletem UV, enquanto no clado de tubo longo *P. axillaris* absorvem UV e *P. exserta* reflete UV (Sheehan *et al.*, 2016).

Os genes que regulam a produção de antocianinas e flavonóis já foram estudados em *Petunia*, tendo sido caracterizados amplamente o gene *AN2,* que controla a produção de antocianina (Quattrocchio *et al.*, 1999; Hoballah *et al.*, 2007), e o fator de transcrição *MYB-FL,* que regula os níveis de flavonóis (Sheehan *et al.*, 2016). Em função dos resultados descritos nestes trabalhos, a hipótese mais provável é que o ancestral polinizado por abelha expressava o gene *MYB-FL* em níveis baixos, pois os flavonóis também são importantes para várias outras funções, mas não o suficiente para permitir a absorção de UV pelas flores. Com uma inserção de 977 pares de base na região reguladora do gene *MYB-FL,* que resultou em um aumento da expressão e acúmulo de flavonóis, esse pigmento passou a competir com a rota de síntese de antocianina por precursores. Esse aumento de flavonóis resultou em flores de *P. axillaris* brancas, absorventes de UV e atrativas para mariposas. Depois disso, uma deleção de 1-bp inativou a função de *MYB-FL* resultando no fenótipo hoje encontrado em *P. exserta*, ocasionando perda de flavonóis, mudança de cor para flor vermelha e de polinização por beija-flores (Sheehan *et al.*, 2016; Grotewold, 2016).

Os estudos sobre o néctar no gênero se concentraram nas espécies *P. integrifolia* e *P. axillaris* e tem mostrado que *P. axillaris* possui uma faixa de volume de 13-23 µl, enquanto *P. integrifolia* possui uma média de 1,2 µl (Stuurman *et al.*, 2004; Galliot *et al.*, 2006; Brandenburg *et al.*, 2009). Essas medidas de néctar estão de acordo com as sugestões de Cruden (1983), que diz que flores polinizadas por morcegos, aves ou mariposas produzem volumes relativamente maiores de néctar em comparação com flores polinizadas por abelhas, borboletas ou pequenas mariposas.

Assim como o néctar, as pesquisas para identificar os polinizadores efetivos se concentraram nas espécies *P. integrifolia* e *P. axillaris*. *P. integrifolia* é polinizada por abelhas solitárias dos gêneros *Callonychium* (Wittmann *et al.* 1990), *Calliopsis,* and *Leioproctus* (Gübitz *et al.* 2009). Gübitz *et al.* (2009) observando indivíduos de *P. axillaris* no Uruguai, identificou as espécies de mariposas *Manduca sexta* e *Eumorpha vitis* como polinizadores desta espécie.

3. Aromas em pólen

Uma vez encontrado visualmente as fontes de recurso, alguns grupos de insetos polinizadores utilizam os aromas florais como reconhecimento químico para confirmar a presença do recurso (Dobson, 1994). As flores de *P. axillaris* emitem uma mistura de vários compostos, dominados por metilbenzoato, benzaldeído e álcool benzílico, durante a noite (Hoballah *et al.*, 2005). Esse aroma floral é um sinal para induzir o comportamento alimentar em *Manduca sexta*, uma espécie de mariposas (Raguso & Willis, 2002, 2005). As flores de *P. integrifolia,* polinizadas por abelhas, produzem quantidades comparativamente mais baixas de benzaldeído e apenas vestígios de alguns outros compostos (Hoballah *et al.*, 2005). Em *P. exserta,* as flores são desprovidas de perfumes florais (Klahre *et al.*, 2011).

No que diz respeito à sinalização química para mariposas, é evidente que a composição de aromas tem como finalidade o reconhecimento da espécie que fornece o recurso, que no caso das mariposas é o néctar. Já nas abelhas, sinais visuais ou aromáticos no pólen estão diretamente envolvidos na atração e orientação do polinizador para os órgãos reprodutivos (Dobson, 1994; Dobson & Bergström, 2000), tendo em vista que as abelhas tem como finalidade primordial a coleta de pólen. Em *Petunia* ainda estão sendo produzidos trabalhos especificamente na decomposição do aroma envolvido no reconhecimento do pólen. O primeiro e único trabalho a tentar qualificar os compostos do pólen em *Petunia* foi Verdonk *et al.* (2003), onde detectaram metilbenzoato e benzaldeído (anteras com pólen exposto) em *P. axillaris*.

4. Biologia reprodutiva e fluxo gênico em *Petunia*

Petunia é um gênero que envolve espécies tanto com sistemas de autocompatibilidade como de autoincompatibilidade. Todas as espécies do clado tubo curto são consideradas auto incompatíveis (Tsukamoto *et al.*, 1998; Stehmann *et al.*, 1999; Kokubun *et al.*, 2006), enquanto que *P. occidentalis* (Tsukamoto *et al.* 1998), *P. exserta* (Stehmann *et al.* 1999; Watanabe *et al.* 2001), *P. secreta* (Stehmann *et al.* 1999) são auto compatíveis. Em *P. axillaris* sistemas de compatibilidade diferentes podem ser encontrados, algumas vezes em uma mesma subespécie. Nas subespécies *parodii* e *subandina* as populações estudadas foram autocompatíveis; enquanto a subespécie *axillaris* possui linhagens autocompatíveis e

autoincompatíveis (Tsukamoto *et al.*, 1998; Kokubun *et al.*, 2006; Turchetto *et al.,* 2014).

Assim podemos afirmar que as espécies do clado tubo curto e as linhagens de *P. axillaris* que são autoincompatíveis são alógamas, pois seu sistema de incompatibilidade garante a fecundação cruzada. Já nas espécies autocompatíveis, observa-se um sistema misto de cruzamento, com a predominância de autopolinização ou polinização cruzada dependente das condições morfológicas ou desempenho do polinizador. Por exemplo, em *P. exserta* existe hercogamia entre as estruturas reprodutivas (onde os estames estão abaixo do estigma) (Stehmann *et al.*, 1999), favorecendo uma situação em que o polinizador promova a fecundação cruzada. Porém essa hercogamia é temporária, pois os estames continuam crescendo e ultrapassam o estigma, quase sempre promovendo uma autopolinização espontânea. Nas espécies *P. secreta* e *P. axillaris* não existe polinização espontânea, pois as anteras permanecem sempre abaixo do estigma, logo, a dependência do polinizador determina qual sistema será favorecido, autopolinização ou polinização cruzada.

Não há isolamento reprodutivo entre as espécies do gênero, pois híbridos artificiais já foram produzidos entre todas elas (Watanabe *et al.*, 1996; Ando *et al.*, 2001; Watanabe *et al.*, 2001) e diversos híbridos naturais já foram descritos (Lorenz-Lemke *et al.,* 2006; Segatto *et al.,* 2014; Turchetto *et al.,* 2015). As espécies florescem de modo geral no mesmo período, não havendo isolamento temporal (Stehmann *et al*., 2009). Nas espécies do clado tubo curto, que são auto incompatíveis e alopátricas, os polinizadores naturais são compartilhados (Silva, 1994) e cruzamentos interespecíficos artificiais resultam em híbridos férteis (Watanabe *et al.*, 1996), sugerindo que o isolamento geográfico é o principal fator envolvido na manutenção da integridade dessas espécies (Stehmann *et al.*, 2009). Apesar da falta de barreiras reprodutivas e o fato de que as espécies *P. integrifolia*, *P. exserta* e *P. axillaris* são simpátricas em diversas localidades, existe entre elas mecanismos de isolamento ecológicos (Ando *et al.*, 2001; Dell'Olivo *et al*., 2011) que previne o fluxo gênico interespecífico, como por exemplo, as adaptações para polinizadores diferentes, o que explicaria a rara incidência de hibridação na natureza (Lorenz-Lemke *et al.,* 2006; Segatto *et al.,* 2014).

5. *Petunia secreta*

A espécie *Petunia secreta* é endêmica da Serra do Sudeste, Rio Grande do Sul, ocorrendo na localidade denominada "Pedra do Segredo". A Pedra do Segredo é, na verdade, um conjunto de cerros formados por conglomerados e arenitos ferruginosos, localizado no

município de Caçapava do Sul. Geralmente formadas por poucos indivíduos, as populações desta espécie podem ser encontradas em afloramentos rochosos expostos ao sol (Stehmann & Semir, 2005; Turchetto *et al.,* 2016).

Petunia secreta é filogeneticamente relacionada às espécies *P. axillaris* e *P. exserta* (Kulcheski *et al.*, 2006; Fregonezi *et al.,* 2013; Reck-Kortmann *et al.,* 2014), compartilhando com elas uma série de características morfológicas, como o hábito ereto ou ascendente, folhas basais e apicais com tamanhos e formas diferentes (heterofilia), tubo da corola longo e hipocrateriforme, pólen amarelo, pedúnculos frutíferos eretos com cápsulas grandes (mais de 9 mm de comprimento) com muitas dezenas de pequenas sementes (com menos de 0,5 mm de comprimento) (Stehmann & Semir, 2005; Stehmann *et al.*, 2009).

Observações realizadas em uma população de *P. secreta* mostraram que esta espécie é visitada por abelhas do gênero *Pseudagapostemon* sp. (Stehmann & Semir, 2005). *Petunia secreta* tem um tubo estreito, tornando o néctar inacessível às abelhas com língua curta (Fregonezi *et al.*, 2013). Esta característica morfológica reforça a ideia de que esta espécie foi secundariamente ajustada à melitofilia (Reck-Kortmann *et al.*, 2014) e teve sua origem a partir de ancestrais que utilizam polinizadores de língua longa, aptos para alcançar a câmara nectarífera, como *P. axillaris* e *P. exserta* (Stehmann, 1999; Fregonezi *et al.*, 2013).

Recentemente, uma nova população foi descrita para *P. secreta* distante aproximadamente 20 km da região da Pedra do Segredo (Turchetto *et al.,* 2016). Embora propondo se tratar da mesma espécie, estes autores demonstraram a existência de duas linhagens evolutivas distintas, correspondendo cada uma delas a uma das regiões nas quais as plantas de *P. secreta* são encontradas. Neste mesmo estudo, também foi detectada uma alta variabilidade genética para a espécie, tanto quando comparada com as outras espécies do gênero de ocorrência mais ampla como àquelas de ocorrência restrita, contrariando as expectativas citadas na literatura para variabilidade genética em espécies raras como *P. secreta* (Frankham, 2010).

O estudo de *P. secreta* torna-se de relevante interesse, pois a compreensão dos mecanismos envolvidos na relação ecológica com o polinizador, sua dinâmica reprodutiva e modo de fluxo gênico contribuem significativamente para o avanço de estudos evolutivos no gênero *Petunia*. Cada vez mais *Petunia* tem se tornado um modelo de referência para estudos de especiação e os estudos com *P. secreta* serão essenciais para ajudar a contar a história do grupo.

6. Objetivo geral

Identificar o papel da biologia reprodutiva de *Petunia secreta* nos processos de especiação em *Petunia* decorrentes da adaptação à melitofilia, através do estudo da polinização e investigação molecular.

7. Objetivos específicos

7.1 Descrever os atributos florais envolvidos no processo de atração do polinizador em *Petunia secreta*.

7.2 Identificar os visitantes florais e os polinizadores efetivos de *P. secreta*.

7.3 Descrever o processo de polinização de *P. secreta*.

7.4 Identificar o sistema de cruzamento de *P. secreta*.

7.5 Determinar a dinâmica de fluxo gênico via pólen e semente em populações naturais de *P. secreta*.

CAPÍTULO II

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When the old is new: evolutionary trajectory of bee pollination in *Petunia*

Original Article

When the old is new: evolutionary trajectory of bee pollination in *Petunia*

DANIELE M. RODRIGUES[1], CAROLINE TURCHETTO[1], CRIS KUHLEMEIER[2], LORETA B. FREITAS[1,*]

[1]Laboratory of Molecular Evolution, Department of Genetics, Universidade Federal do Rio Grande do Sul, PoBox 15053, Porto Alegre, Brazil

[2]Institute of Plant Sciences, Altenbergrain 21 CH-3013, Bern, Switzerland

*Corresponding author. E-mail: loreta.freitas@ufrgs.br

Running head: Bee attraction in *Petunia*.

Petunia is a young endemic genus from South America that had different colors and shapes of flowers, attracting bees, moths, or hummingbirds as pollinators. The bee-pollinated *P. secreta* is descendent from a moth-pollinated ancestor. The aims of this study were identify the effective pollinator of *P. secreta* and characterize floral traits involved in pollinator attraction. We conducted field observation, collected, recorded flower visitors, evaluated the ultraviolet light response in detached flowers, estimated nectar volume and sugar concentration, and assessed floral pigments to characterize morphological cues for pollinators. The solitary bee *Pseudagapostemon* sp. is the effective pollinator of *P. secreta* and these bees visit flowers only to collect pollen. *P. secreta* petals were reflectant under ultraviolet light; nectar volume varied 4 – 20 µl and sugar concentration was 16 – 26 % per flower; the species presented flavonols and anthocyanins pigments responsible for the corolla color, and yellow pollen. *P. secreta* recovered the ancestral bee-pollination syndrome, turning to the pink flower color and UV-light responses, which enabled this species to attract bees as other species in *Petunia*. Traits as yellow pollen and scent compounds would ensure reproductive isolation when *P. secreta* and other bee-pollinated congeners are in sympatry.

ADDITIONAL KEYWORDS: Anthocyanins – bee-pollination – flavonols – *Petunia* – pollinator attraction – *Pseudagapostemon* – Solanaceae – UV-light response.

INTRODUCTION

Animal pollinators have played a role as drivers of floral diversification and plant speciation (van der Niet & Johnson, 2012; van der Niet, Peakall & Johnson, 2014). For instance, floral differences can affect pollinator attraction and cause premating isolation between species pairs. Different pollinators may drive floral divergence (Fenster *et al.*, 2004), and floral traits, such as shape, color, scent, and phenology, associated with a particular pollinator group are known as floral or pollination syndromes (Faegri & van der Pijl, 1979; Etcheverry & Alemán, 2005).

The evolutionary strategies in specialized pollination systems consist of maximizing the efficiency of specific pollinators that are successful in depositing pollen grains on stigmas and increasing the visitation frequency of conspecific flowers (Reynolds *et al.*, 2009). Thus, the effectiveness of a specialized pollination system depends on the body structure and behavior of the floral visitors as well as the plant attributes that attract them (Roque *et al.*, 2016).

Bees (Hymenoptera: Apoidea: Apiformes) are commonly regarded as highly important pollinators with a diversity of ca. 20 000 species worldwide (Michener, 2007); they are present in most ecosystems and have a long-standing relationship with angiosperms (Michener & Grimaldi, 1988; Engel, 2000; Poinar & Danforth, 2006), playing a key role in ecosystem dynamics (Steffan-Dewenter *et al.*, 2006). These insects use, as a source of flower pollen, nectar or floral oils and are able to identify floral cues at foraging sites that aid them in recognizing food sources (Arenas & Farina, 2014). Indeed, evidence has shown that bee responses to multimodal stimuli (Leonard, Dornhaus & Papaj, 2011; 2012) are intrinsically related (Burger, Dötterl & Ayasse, 2010) and similar to the perception of cross talking between olfaction and vision observed in *Drosophila* (Stewart, Baker & Webb, 2010). Two main features of flowers serve as signals to bees: visual traits, such as the corolla reflecting

ultraviolet (UV) light, and chemical compounds, like the emission of volatile substances (Leonard & Masek, 2014), both features are present in most flowers that emit highly complex species-specific bouquets of volatile organic chemicals (Schiestl, 2010).

The Solanaceae are the most diverse family of euasterids in the Neotropics, with large numbers of endemic species and a remarkable diversity of flower morphology and pollinators (Knapp, 2010). The *Petunia* Juss. is a young genus that belongs to Solanaceae and encompasses 14 species distributed in the subtropical and temperate regions of South America. Molecular studies have suggested the genus has undergone adaptive radiation during the Pleistocene and pollinators contributed to species diversification (Lorenz-Lemke *et al.*, 2010; Fregonezi *et al.*, 2013). With this, the *Petunia* species are known as bee-, bird- or moth-pollinated (Knapp, 2010) and present one-flowered inflorescences with different colors and forms and bilateral flower symmetry (Stehmann *et al.*, 2009). These traits render *Petunia* as a suitable model for pollination-mediated speciation studies (Gübitz *et al.*, 2009; Vandenbussche *et al.*, 2016).

The most recent *Petunia* phylogeny (Reck-Kortmann *et al.*, 2014) exhibited two well-supported clades that are mainly related to the corolla tube length. The first clade includes 11 bee-pollinated species presenting short corolla tubes, pink flowers, and blue pollen. The second clade includes three species with long tubes and yellow pollen (*Petunia axillaris* (Lam.) Britton, Sterns and Poggenb., *Petunia exserta* Stehmann, and *Petunia secreta* Stehmann & Semir) along with different pollinators and corolla colors, plus the sister species *Petunia occidentalis* R. E. Fr., which displays short corolla tube, blue pollen, and is bee-pollinated (all traits that are typical of species included in the first clade). The main differences between the three species with long corolla tubes are related to adaptations to different pollinators - plants of *P. axillaris* have white flowers, produce night floral scent, and are moth-pollinated (Galetto & Bernardello, 1993; Ando *et al.*, 2001); *P. exserta* possesses

red flowers, anthers and stigma emanating from corolla, and are pollinated by hummingbirds (Lorenz-Lemke *et al.*, 2006; Stehmann *et al.*, 2009); and *P. secreta* presents pink flowers and bees are suggested as their pollinators (Stehmann & Semir, 2005).

Several studies, mainly on experimental conditions, have been conducted on floral traits attracting pollinators in *Petunia* species, especially within *P. axillaris*, *Petunia inflata* R. E. Fr*., Petunia integrifolia* (Hook.) Schinz and Thell*,* and *P. exserta,* considering flower morphology, scent emission, nectar composition, and UV-light responses (see an overview in Sheehan, Hermann & Kuhlemeier, 2012). These studies included analyses of chemical composition of aroma and nectar (Sheehan *et al.*, 2012; Gleiser *et al.*, 2014), molecular characterization of genes (Hermann & Kuhlemeier, 2011; Klahre *et al.*, 2011; Hermann *et al.*, 2015; Sheehan *et al.*, 2016), and a number of comparisons of pollinator behavior and preferences (Hoballah *et al.*, 2007; Venail, Dell'Olivo & Kuhlemeier, 2010; Dell'Olivo & Kuhlemeier, 2013). Despite these previous inquiries, the literature on reproductive biology, pollinator attraction, and evolutionary aspects of plant-insect interactions remains scarce for the majority of *Petunia* species in nature.

Petunia secreta is an annual species that blooms from September to December (spring season in the South Hemisphere), presenting pink flowers with long and salveform (a tubular-shaped corolla with a flat expanded limb) corolla tubes and yellow pollen. The fruit-stalk is erect with large capsules (9–17 mm X 6–8 mm) containing hundreds of seeds (Stehmann & Semir, 2005). *Petunia secreta* is a rare species found within two landscapes - one is the restricted area called Pedra do Segredo (Fig. 1A) where plants grow exposed to the sun on the top of conglomerate sandstone towers that are equal to between 300-400 m in elevation (Stehmann *et al.*, 2009) and the other is an adjacent area in the municipality of Caçapava do Sul, Rio Grande do Sul, southern Brazil, in an open vegetation flat area along roads of ca. 20-30 km distance from Pedra do Segredo (Turchetto *et al.*, 2016). Two different evolutionary

lineages were recognized associated to these different environments (Turchetto *et al.*, 2016). The original description of *P. secreta* reported solitary bees visiting the flowers (Stehmann & Semir, 2005), though this work did not include any systematic observation of pollination.

In the present work, we sought to characterize certain floral attributes that favored pollinator attraction as well as determine the effective pollinator of *P. secreta* and contribute to understanding the evolutionary scenario of diversification in *Petunia* genus.

MATERIAL AND METHODS

PLANT MATERIAL

During the spring for two years (September to December, 2014 and 2015), we visited the region of occurrence of *P. secreta* (Fig. 1A) to observe pollinators and sampling seeds. We germinated seeds in growth chambers and cultivated plants in a greenhouse to obtain nectar in order to perform stigmatic receptivity testing, UV light testing, and to quantify floral pigments with the goal of not impacting natural populations. For every collection site and analyzed species, we made exsiccates (Table S1).

For comparison, during UV light response experiments, we used flowers from greenhouse grown plants of *P. axillaris, P. exserta,* and *P. inflata,* and to quantify floral pigments we employed petals of cultivated individuals of *P. axillaris*, *P. exserta,* and *P. integrifolia* (Table S2).

FIELD OBSERVATIONS

We observed the floral visitors and potential pollinators in nature on flowers of *P. secreta* individuals from the Pedra do Segredo locality (30º 32' 47" S 53º 33' 03" W) (Fig. 1A). In total, 176 hours of observation for 23 different flowers was conducted. The observations took place over 22 days (12 days in 2014; 10 days in 2015) at nine hours per day (08:00 AM to

05:00 PM). We photographed and filmed all visitors and pollinator behavior using a Nikon D3200 SLR camera with Nikon DX AF-S Nikkor18-55 mm micro lens (Nikon Co., Tokyo, JA) positioned three meters from the flowers to reduce the interference of observer presence. We registered the number and taxonomic group of visitors per flower and the kind of behavior (landing site in bloom, contact with pollen, location of accession of pollen grains in pollinator, ability to touch the stigma, visit duration, collected floral resource type, number of visited flowers, and pollinator behavior during the visit). We considered as effective pollinators all floral visitors who mandatorily presented body structure where the pollen was adhered, touched the stigma, and repeated this sequence at 90% or more in visits. Potential pollinators were collected and preserved in 70% ethanol to taxonomical identification. The bees were identified and deposited into the collection of the Museum of Science and Technology of the Pontifícia Universidade Católica do Rio Grande do Sul (Rio Grande do Sul, Porto Alegre, RS, Brazil).

NECTAR EVALUATION AND STIGMATIC RECEPTIVITY

We measured volume and sugar concentration of nectar in 20 flowers collected from four distinct individuals of *P. secreta* (five flowers per individual) grown in the greenhouse. The flower buds were bagged and all nectar volume were extracted 24 hours after the opening flower with a graded 25 µl volume Hamilton micro syringe (Sigma-Aldrich Co, St. Louis, Mo, USA). To measure the sugar concentration, the total volume of extracted nectar was trickled on a portable refractometer. Stigma receptivity was tested by plunging the stigmatic surface into hydrogen peroxide P.A. (Merck & Co, Kenilworth, NJ, USA) at 100% in four stages, 10 flowers per stage, in five individuals: pre-anthesis floral buds; flowers after anthesis immediately after the opening of the anthers; in the beginning of the corolla color changing (pink change to purple, indicating early flower senescence stage); and flowers with

wilted petals. It was considered a positive result in the presence of oxygen bubbles resulting in stigma-hydrogen peroxide reactions (according to Zeisler, 1938).

Ultraviolet (UV) light response

We obtained images of detached flowers with UV light using a Nikon 60 mm 2.8D micro lens with a Nikon D7000 SLR camera that was converted to record UV light by replacing the manufacturer's filter with a UV-specific filter that blocked visible and infrared light (Advanced Camera Services Ltd, Watton, UK). A Metz MZ76 flashgun (Metz-Werke GmbH & Co KG, St. Chandler, AZ, USA) that was modified to produce UV-A light (320-390 nm; Advanced Camera Services Ltd.) provided the light source. Images were converted to grey scale in Photoshop CS5 (Adobe Systems Co., San Jose, CA, USA) and, where necessary, exposure was adjusted over the complete image. Flowers were scored either as UV-absorbent or UV-reflective based on comparison with the positive control UV-absorbent *P. axillaris* flower.

Spectrophotometric quantification of flavonols and anthocyanins

We sampled 8 mm diameter discs from the corolla limb of three flowers (different individuals) for each of the four species to quantify flavonols and anthocyanins. We put each disc into 1 mL of extraction buffer (2:1:7 methanol: acetic acid: water) and kept the solution for 48 h in the dark (modified from Ando *et al.,* 1999). Further, we used a spectrophotometer SpectraMax M4 (Carl Zeiss AG, Oberkochen, DE) to measure the absorption spectra. Flavonols represented summed absorbance values over 315-378 nm whereas anthocyanins demonstrated a range of summed absorbance values between 445-595 nm. Comparisons were made among plants growing under the same conditions.

RESULTS

FIELD OBSERVATIONS

Petunia secreta flowering was present throughout September to December in both years and fruit ripening was concentrated in November. Flower opening was during the daytime and no set period was observed. The flowers remained open ca. two days if the pollen contacted the stigma and began senescence after four days in the absence of pollination. Anther dehiscence took place concomitantly with flower opening (see Fig. S1 for more plant details).

During the 176 hours of observation, three different genera of bees and one non-identified hummingbird species were recorded as flower visitors (Table 1). The bees were established as belonging to the *Pseudagapostemon* genus (Fig. 1B) that landed directly on the reproductive structures and collected pollen exclusively (these individuals approached the flowers, flew away, and approached again, several times, until landed); four male bee individuals of the species *Lanthanomelissa clementis* were seen only once after 06:00 PM, grouped and using the flower as a dormitory and remained inside the flower until the following morning; one bee individual of the species *Xylocopa* sp. that appeared in October 2015 in just one day, visited the flowers several times, and cut out a piece of the corolla and took it away (Movie S1). The non-identified hummingbirds were seen once in October and four times in November 2015 (upon each visit, the birds introduced the bill into the flower within fractions of seconds strictly on one flower per visit). We were not able to take photos that would foster identification of the species of hummingbirds. Several butterflies were observed flying close to the flowers, but did not show interest.

Bees of *Pseudagapostemon* sp. were the only visitors that presented a constant frequency of visits and displayed behavior and body structure that fulfilled all our criteria for consideration to be the effective pollinator of *P. secreta*. We observed 22 visits of *Pseudagapostemon* sp. individuals that landed directly on the reproductive structures (Fig.

1C), with the front legs scraping the anthers and transferring pollen to the scopa in the abdomen (dense set of hair or bristles specialized to pollen adherence) and to the hind tibia (Movie S2), always positioned on the flower with the abdomen and legs in front of the stigma (Fig. 1C). These individuals took, on average, two minutes to collect all pollen and exclusively processed it. *Pseudagapostemon* sp. individuals were observed on *P. secreta* flowers only when pollen was present and, therefore, each flower received a maximum of two visits. The differences in anthers' height (Fig. 1D) influenced bee behavior during pollen collection, making the insect stand in different directions and thus sloped across from the stigma surface so as to completely remove pollen. There was no standard day-period for the visit of *Pseudagapostemon* sp. individuals on *P. secreta* flowers; the visits were spread throughout the daytime. Visits were more frequent on sunny days and none were conducted on rainy, windy or cloudy days. After approaching the flowers, bees flew around the flower, approaching and moving away several times. In the presence of pollen, bees aligned and removed all pollen; in the absence of pollen, bees did not land.

FLORAL ATTRACTION AND REWARDS

The nectar total volume ranged among 4 – 20 µL per flower (mean 8 µL) and the sugar total volume varied among 16 – 26% per flower (mean 21.5%) among the 20 evaluated flowers (Table S3), while the stigma receptivity tests revealed that in the 50 analyzed flowers, the stigma surface was active during all stages, suggesting that *P. secreta* stigma is receptive before flower opening and as following it until the petal withered if not pollinated.

Flowers of species *P. axillaris*, *P. exserta*, *P. inflata,* and *P. secreta* differed in appearance under visible (Fig. 2A) and UV light (Fig. 2B). The responses to UV light revealed that, as expected for flowers pollinated by moths, *P. axillaris* petals absorbed UV light (dark color); the petals of *P. inflata* and *P. secreta* reflected UV light (light color), a

response that is attributed to bee-pollinated flowers; and the petals of *P. exserta* also reflected UV light (light color).

We quantified the flavonols and anthocyanins in petals of completely opened flowers of *P. axillaris, P. exserta*, *P. integrifolia,* and *P. secreta* and observed the presence of flavonols in the four species with wavelength ranges of 302 – 340 nm; *P. axillaris* and *P. secreta* exhibited higher values of absorbance (1.19 and 1.15, respectively), whereas *P. exserta* and *P. integrifolia* demonstrated 0.38 and 0.58, respectively (Fig. 2C). Anthocyanin peaks appeared in *P. exserta*, *P. secreta,* and *P. integrifolia* with wavelengths between 524 – 538 nm; absorbance values were species specific (0.56, 0.36, and 0.10, respectively) and *P. axillaris* did not present anthocyanins (Fig. 2C).

DISCUSSION

Evidence based on phylogenetic approaches has shown that shifts in pollinators are common, being associated with multiples divergence events (van der Niet & Johnson, 2012); however, these approaches alone are not adequate for distinguishing among several pollinator-driven evolutionary processes (Knapp, 2010). The flowers of a particular plant species usually are visited by various groups of pollinators (Potts *et al.*, 2010) and these floral visitors have different abilities to pollinate the flowers (Rosas-Guerrero *et al.*, 2011). Fenster *et al.* (2004) proposed that several floral traits reflected the adaptation to specific pollinators that visit flowers most frequently and efficiently. Thus, specificity between characteristics of flowers and pollinator behavior or morphologies may contribute to maintaining pollination restricted to individuals of the same species and enhancing reproductive isolation in a variety of plant species (Scopece, Schiestl & Cozzolino, 2014; Breitkopf *et al.*, 2015).

Here, we investigated the pollination system in *P. secreta,* a rare and narrow endemic species belonging to the *Petunia* genus. *Petunia* phylogeny exhibits evidence for bee-

pollination and morphological traits associated with melittophily as the ancestral state within the genus (Kulcheski *et al.*, 2006; Reck-Kortmann *et al.*, 2014). Ancestral reconstruction suggests a purple and short corolla tube, UV reflectivity, blue pollen, and non-scent producing individuals originating in the low lands from the Pampas region in Uruguay, Rio Grande do Sul Brazilian state, and the Pampa Argentinean Province where *P. integrifolia* species complex grew (Reck-Kortmann *et al.*, 2014) between 1.3 (Lorenz-Lemke *et al.*, 2010) and 2.8 million years ago (Särkinen *et al.*, 2013). Eleven *Petunia* species present characteristics similar to the common ancestral form grouped into the clade I in the genus phylogeny (see Fig. 2A; Reck-Kortmann *et al.*, 2014), whereas into the clade II, species with long corolla tube diverge in flower morphology and pollinators and *P. secreta* and *P. axillaris* subsp. *axillaris* share the most recent ancestor (Fig 2A; Fig. S2). Nectar volume varies among species of *Petunia*, though the sugar concentration was generally high (Gübitz *et al.*, 2009).

Oligolectic bees *Callonychium petuniae* are believed to have coevolved with *P. integrifolia*, and so bee and plant traits would therefore be related, such as the color of the unscented flowers and bee spectral receptor cells that are sensitive to UV, blue, green, and red light (Cure & Wittmann, 1990; Wittmann *et al.*, 1990). These authors showed that flowers of *P. integrifolia* not only serve as food resources for the bees, but also as mating sites. Females of *C. petuniae* collect pollen and male's searching behaviors for mates usually results in nights being spent inside flowers waiting for females. Males therefore contribute to pollination because they collect nectar and cut the stamens. As the flowers wither after roughly two days, males contribute much more to pollination than females during early spring (Wittmann *et al.*, 1990). Besides *C. petuniae*, Ando *et al.* (2001) observed species of the *Leiproctus* subgenus *Hexantheda* collecting pollen and consuming nectar while thrusting their whole bodies into the corolla tube of *P. integrifolia*. Moreover, these authors described a

synchronized opening and closing movement of the corolla lobes in *P. integrifolia* and the active flight period of the bees.

Petunia secreta flowers possess pink long corolla tubes and yellow pollen, and the species belongs to the clade II (Reck-Kortmann *et al.*, 2014) in *Petunia* phylogeny (Fig. 2A). The species in clade II represent the highest floral diversification in the genus probably driven by pollinator selection (Fregonezi *et al.*, 2013). *Petunia secreta* is the sister species of the large and white *P. axillaris* subsp. *axillaris* that also has a long, slender, sub-cylindrical corolla tube, but emits a strong fragrance towards the evening and absorbs UV light (Fig. 2B) and is frequently found close to the *P. secreta* occurrence area (Turchetto *et al.*, 2014; 2015). The characteristics present in *P. axillaris* are well-known attributes that attract hawkmoths (Ando *et al.*, 1995; Venail *et al.*, 2010; Klahre *et al.*, 2011) and the subspecies of *P. axillaris* are widespread in the South American Pampas (Turchetto *et al.*, 2014), while *P. secreta* grows in a small geographic area on the border of *P. axillaris* subsp. *axillaris* distribution. The major determinant of flower color variation between *P. integrifolia* and *P. axillaris* that causes the major shift in pollination biology is the *ANTHOCYANIN2* gene (Hoballah *et al.,* 2007) and includes inactivation on gene function promoting change in corolla color from pink to white. Between *P. axillaris* and *P. exserta*, a tight genetic linkage of loci specifies the five major pollination syndrome traits that differentiate these species (Hermann *et al.,* 2013). Similar genetic mechanisms could be involved in changes that render bee-pollination to *P. secreta*.

Our findings demonstrated that *Pseudagapostemon* sp. bees are the most effective pollinators of *P. secreta* (Fig. 1B-C; Movie S2), confirming previous observations (Stehmann & Semir, 2005). The interaction between *Pseudagapostemon* sp. bees and *P. secreta* seems to be different that was observed between *P. integrifolia* and *Callonychium petuniae* (Wittmann *et al.*, 1990) or between *P. integrifolia* and *Leiproctus* subgenus *Hexantheda* (Ando *et al.*,

2001). While in *P. secreta* the pollen is the only reward, in *P. integrifolia* (a species that grows in a geographical area close to *P. secreta* distribution), bees collect pollen and also obtain nectar that is deposited in the bottom of the short, roomy, and shallow corolla tube. Despite *P. secreta* flowers producing nectar, bees cannot approach the bottom of corolla tube to collect it (Fig. 2D). Field observations (data not shown) and previously published measures (Stehmann & Semir, 2005; Turchetto *et al.*, 2016) indicate that the distance between the point where the fillets weld at the corolla to the deeper portion of the tube where the nectar accumulates is ca. 2 cm (Fig. 2D) and the tube along this region is slender (ca. 2-4 mm in diameter). These measurements suggest that it is impossible for individuals of *Pseudagapostemon* sp. to gather the nectar, and this is different from *Callonychium* individuals that can obtain nectar in flowers of *P. integrifolia* (Wittmann *et al.*, 1990). The body length of *Pseudagapostemon* sp. is ca. 8 mm and no records were found on the proboscis extension, however even if the tongue has the same body size (five to 11 mm described for the genus; Michener, 2007), it would still not cover the distance of 20 mm to the nectar in *P. secreta*. Furthermore, during all observations, the *Pseudagapostemon* sp. individuals did not display nectar collection behavior that demands more time and diverse movements than those animals presented.

Several studies have proposed that bees use a combination of visual, olfactory, and tactile floral cues to find appropriate host plants (Dobson, 1987; Whitney *et al.*, 2009; Dötterl & Vereecken, 2010; Schiestl, 2010; Leonard & Masek, 2014; Milet-Pinheiro, Ayasse & Dötterl, 2015; Ruedenauer, Spaethe & Leonard, 2015) and that these insects are capable of learning floral signals during foraging bouts (Zhang *et al.*, 2006; Muth, Francis & Leonard, 2016a). A general pattern may emerge from these works, being that during early bee trips, they rely primarily on olfactory cues and that the visual cues become more important in host-plant location as bees become more experienced (Döterl & Vereecken, 2010; Zhang *et al.*,

2016). *Petunia secreta* flowers have a pink corolla that reflects UV light (Fig. 2B), also appearing in the ancestral state of the *Petunia* genus (Fig. 2A; Reck-Kortmann *et al.*, 2014). These cues might serve as the main visual stimuli for long-distance attraction and can help bees find host plants. On the other hand, the behavior of *Pseudagapostemon* sp. bees when approaching *P. secreta* flowers (flying in circles ca. 10 cm from the corolla and leaving if the anthers do not have pollen) is a signal that certain pollen compounds constitute a stimulus when bees land on the flowers. Thus, we hypothesize that a number of pollen components play a key role in this species-specific interaction, such as proteins detected by their color or odor because pollen is the primary source of protein for bees (Muth, Papaj & Leonard, 2016b). Indeed, different sets of volatile pollen compounds were present when comparing *P. secreta* and *P. integrifolia* (D. M. Rodrigues, unpublished data). The critical nature of floral scent in the foraging behavior of host-specialized solitary bees has been demonstrated in honeybees that associate scent and pollen (Arenas & Farina, 2012). These volatile emissions may help guide bees to unpollinated flowers, increasing plant fitness and bee energetic returns (Rodriguez-Saona *et al.*, 2011). Pollen-specific odors are especially important because they allow bees to detect pollen availability from a long distance before landing (Dobson & Bergström, 2000; Dötterl *et al.*, 2005; Dötterl & Vereecken, 2010). Pollen color is a key difference between *P. secreta* and other bee-pollinated species of *Petunia* (Fig. 2A) and it is known that pollen-foraging bees can recognize visual cues, like color, and remember them (Muth *et al.*, 2016b).

The results presented in this work show that *P. secreta* produces nectar and, despite it not acts as a reward for the most effective pollinator (*Pseudagapostemon* sp.), it could be used by other potential pollinators that exist in the same area and effectively pollinate other *Petunia* species. The large, white flowers of *P. axillaris* subsp. *axillaris* reward hawkmoth pollinators with nectar containing glucose, fructose, and sucrose, though no amino acids

(Brandenburg, Kuhlemeier & Bshary, 2012), secreted continuously throughout the flower's lifetime (Galleto & Bernardello, 1993) and deposited at the base of the corolla. In nature, flowers of *P. axillaris* subsp. *axillaris* produce a mean of 10 μL of nectar with a 35% sugar concentration (Gleiser *et al.*, 2014), close to the optimal nectar volume and sugar concentration reported for hawkmoths and several types of bees (Kim, Gilet & Bush, 2011). The average volume and sugar concentration of *P. secreta* nectar was lower than that observed in *P. axillaris* subsp. *axillaris* (8 μL and 21.5%, respectively). So, we therefore suggest that these nectar characteristics, summed up as flower color, UV response, and absence of scent, are not attractive to the *Manduca* sp. in *P. secreta* despite hawkmoths occurring in the *P. secreta* area (Ando *et al.*, 2001) and effectively pollinating *P. axillaris* (Ando *et al.*, 1995; Venail *et al.*, 2010; Klahre *et al.*, 2011).

Petunia exserta flowers present red and bright corollas that are capable of attracting birds. The petal limbs in this species are backward-folded and the nectar is abundant, accumulating in a long and narrow floral tube. The reproductive structures are exserted from the corolla, which improves contact with a bird's head and better facilitates pollen transfer (Lorenz-Lemke *et al.*, 2006; Stehmann *et al.*, 2009). To date, there have been no reports on nectar volume or concentrations in plants of *P. exserta*. In our study, hummingbirds were observed close to *P. secreta* flowers in very low frequency (Table 1) and their behavior was clearly prospective (introduced the bill into flowers for just fractions of seconds). Combining bird behavior in *P. secreta* flowers with the flower structure in this species, it is improbable that hummingbirds act as pollen vectors for this *Petunia* species (Table 1; Fig. 1D). Several studies have described hummingbirds exhibiting optimal licking in plant species with nectar concentrations consisting of approximately 35-40 % sucrose and in large volumes (Kingsolver & Daniel, 1983; Kim *et al.*, 2011). However, the hummingbird-pollinated plant species *Elleanthus brasiliensis* (Lindl.) Rchb.f. and *E. crinipes* Rchb.f. present similar sugar

concentrations to those found in *P. secreta* (Nunes *et al.*, 2016). Future investigations of nectar composition in *P. secreta* and *P. exserta* are required to better understand why hummingbirds do not, at the least, collect it in *P. secreta*.

The pollination systems of the *Petunia* genus have been defined mainly through floral traits, such corolla color and morphology, nectar content and composition, and scent production (Stuurman *et al.*, 2004). Recently, one molecular study elucidated the transitions in UV absorbance from bee to moth pollination and from moth to hummingbird in *Petunia,* determining that mutations in a single gene (*MYB-FL*) are responsible for the pollinator changes (Sheehan *et al.*, 2016). Similar molecular mechanisms could explain the response to UV light in *P. secreta*. Examinations focusing on genetic loci associated with floral traits in *Petunia* have shown that the genomic architecture of pollination syndromes allows rapid responses to pollinator availability changes (Klahre *et al.*, 2011; Hermann *et al.*, 2013; 2015). However, the genetic of the specific interaction between *P. secreta* and their pollinators has not yet been looked into.

Plants of *P. secreta* grow in a small region where other *Petunia* species also occur and, certainly, the different rewards among them and the specific morphological attributes, in addition to ecological differences, prevent interspecific crossing, contributing to reproductive isolation and speciation. Under experimental conditions, at least, all *Petunia* species preserve inter-crossing ability (Watanabe *et al.*, 2001) and previous studies have reported that a shift in certain characteristics influence pollinator foraging and contribute to the maintenance of reproductive isolation in the genus (Quattrochio *et al.*, 1999; Ando *et al.*, 2001; Galliot, Stuurman & Kuhlemeier, 2006).

In conclusion, *P. secreta* exhibited a set of traits that enable these plants to attract solitary bees as effective pollinators. A number of these characteristics, especially the color of petals, represent a reversion to the ancestral condition in the *Petunia* genus, whereas others,

such as pollen color and probably pollen scent, are novelties that have permitted this species to find a new pollinator species, making reproductive isolation certain among congeneric species distributed within the same area. Developing molecular characterization of the potentially involved genes and expanding studies on gene flow and the reproductive biology underlying *P. secreta* will definitely provide new insights on speciation of this charismatic genus, serving as a model for other plant species from a phylogenetic point of view.

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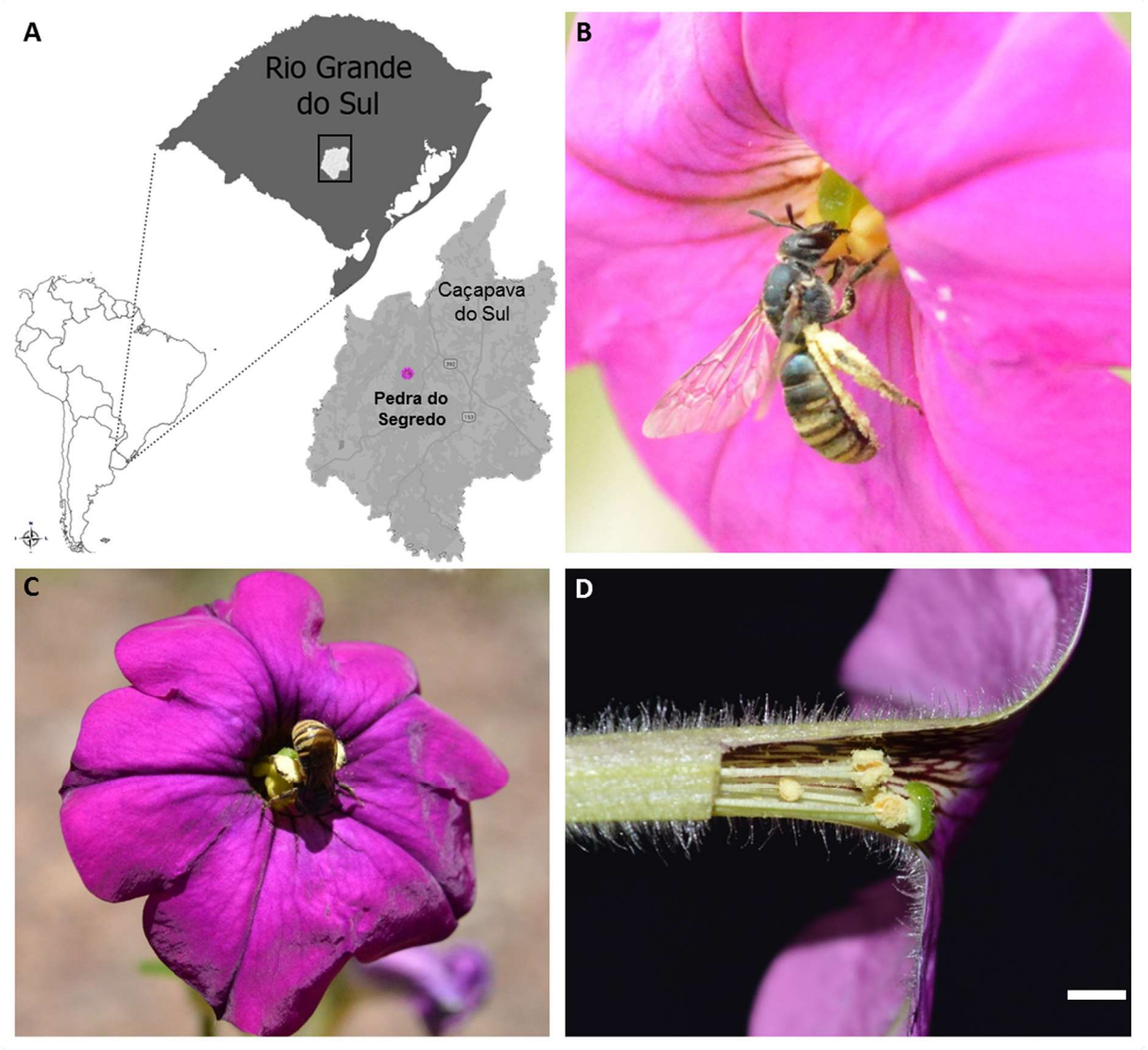

Fig. 1 *Petunia secreta*: A, Collection location; B, Effective pollinator (*Pseudagapostemon* sp. bee) on flower; C, *Pseudagapostemon* sp. positioning for effective pollination of *P. secreta*; D, Reproductive organs of *P. secreta* highlighting the anthers and stigma position and differences in anthers' length. Bar = 1 cm.

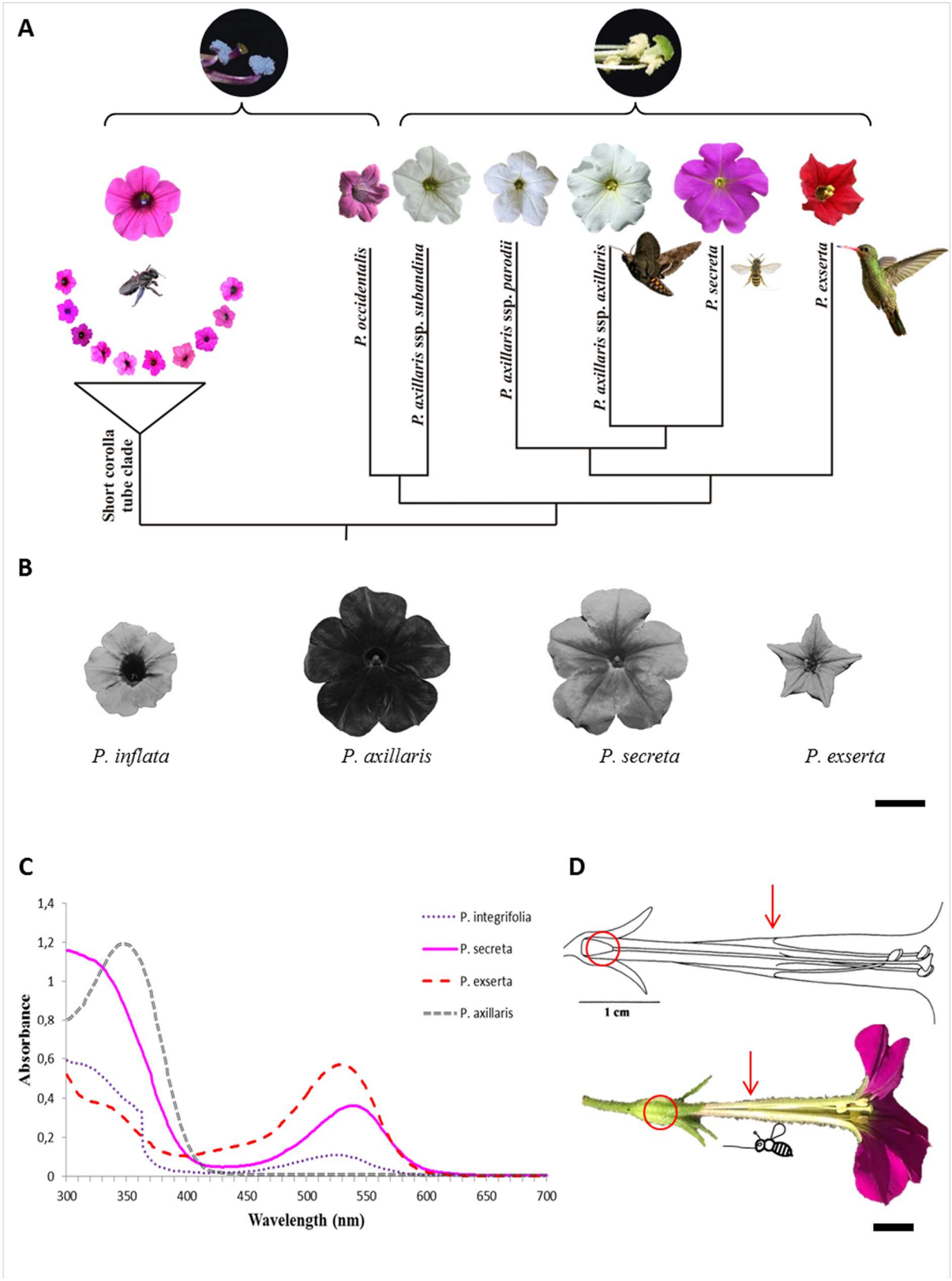

Fig. 2 Pollinator attraction cues: comparison among *Petunia* species. A, Phylogeny of the genus *Petunia* exhibiting the relationships of corolla colors and pollinators (adapted from Reck-Kortmann *et al*., 2014); B, UV light responses in four *Petunia* species. Detached

flowers were exposed to UV light and different responses were observed among species (UV absorbing = dark flowers; UV reflecting = light flowers). A flower of *P. inflata* represents the short corolla tube clade; C, Pigment components of petals in *Petunia* species. Different peaks represent different pigment chemical classes according the wavelength range and the lines correspond to different species (see legend); D, Tube length and nectar position. Circle corresponds to the location of nectar accumulation and arrow indicates the point where anther filaments start to weld to the floral tube and form the compartment for the style (linear design of the flower was adapted from Stehmann & Semir, 2005). Bar = 1 cm.

Table 1. Records of floral visitors in *Petunia secreta* from Pedra do Segredo (30º 32' 45.9" S 53º 33' 00.9" W) carried out in two springs.

Year	Day	Hour	Floral Visitor	Behavior
2014	Out 08	13:18	*Pseudagapostemon* sp.	Collected pollen
		14:04	*Pseudagapostemon* sp.	Flew over flowers
		14:20	*Pseudagapostemon* sp.	Flew over flowers
		14:43	*Pseudagapostemon* sp.	Flew over flowers
		15:17	*Pseudagapostemon* sp.	Flew over flowers
		15:59	*Pseudagapostemon* sp.	Flew over flowers
		16:21	*Pseudagapostemon* sp.	Flew over flowers
		16:33	*Pseudagapostemon* sp.	Flew over flowers
		17:16	*Pseudagapostemon* sp.	Flew over flowers
		17:42	*Pseudagapostemon* sp.	Flew over flowers
		17:56	*Pseudagapostemon* sp.	Flew over flowers
		18:10	*Lanthanomelissa clementis*	Slept in flower
	Out 09	08:38	*L. clementis*	Slept in flower
		10:45	*Pseudagapostemon* sp.	Collected pollen
		11:08	*Pseudagapostemon* sp.	Flew over flowers
		11:50	*Pseudagapostemon* sp.	Flew over flowers
		13:13	*Pseudagapostemon* sp.	Collected pollen
		13:21	*Pseudagapostemon* sp.	Flew over flowers
	Out 23	10:40	*Pseudagapostemon* sp.	Collected pollen
		11:08	*Pseudagapostemon* sp.	Collected pollen
		14:56	*Pseudagapostemon* sp.	Flew over flowers
		15:16	*Pseudagapostemon* sp.	Flew over flowers
	Nov 04	13:22	*Pseudagapostemon* sp.	Collected pollen
	Nov 06	10:37	*Pseudagapostemon* sp.	Collected pollen
	Nov 26	11:57	*Pseudagapostemon* sp.	Collected pollen
		13:16	*Pseudagapostemon* sp.	Flew over flowers
		16:43	*Pseudagapostemon* sp.	Flew over flowers
2015	Out 08	14:08	*Pseudagapostemon* sp.	Collected pollen
	Out 21	14:51	*Pseudagapostemon* sp.	Collected pollen
	Out 22	15:12	*Pseudagapostemon* sp.	Collected pollen
		15:18	Hummingbird	Introduced bill in flower
		17:01	*Xylocopa* sp.	Cut a corolla piece
		17:07	*Xylocopa* sp.	Cut a corolla piece
		17:11	*Xylocopa* sp.	Cut a corolla piece
		17:16	*Xylocopa* sp.	Cut a corolla piece
		17:27	*Xylocopa* sp.	Cut a corolla piece
	Nov 03	16:26	*Pseudagapostemon* sp.	Collected pollen
	Nov 06	13:51	*Pseudagapostemon* sp.	Collected pollen
		13:57	*Pseudagapostemon* sp.	Collected pollen
		16:46	Hummingbird	Introduced bill in flower
	Nov 07	12:24	*Pseudagapostemon* sp.	Collected pollen
		12:26	*Pseudagapostemon* sp.	Collected pollen
		12:30	*Pseudagapostemon* sp.	Collected pollen
	Nov 23	15:23	*Pseudagapostemon* sp.	Collected pollen
	Nov 25	11:23	*Pseudagapostemon* sp.	Collected pollen
		11:29	*Pseudagapostemon* sp.	Collected pollen
		12:23	*Pseudagapostemon* sp.	Collected pollen
		13:39	*Pseudagapostemon* sp.	Collected pollen
		13:36	Hummingbird	Introduced bill in flower
		14:11	Hummingbird	Introduced bill in flower
		14:59	Hummingbird	Introduced bill in flower

Supporting Information

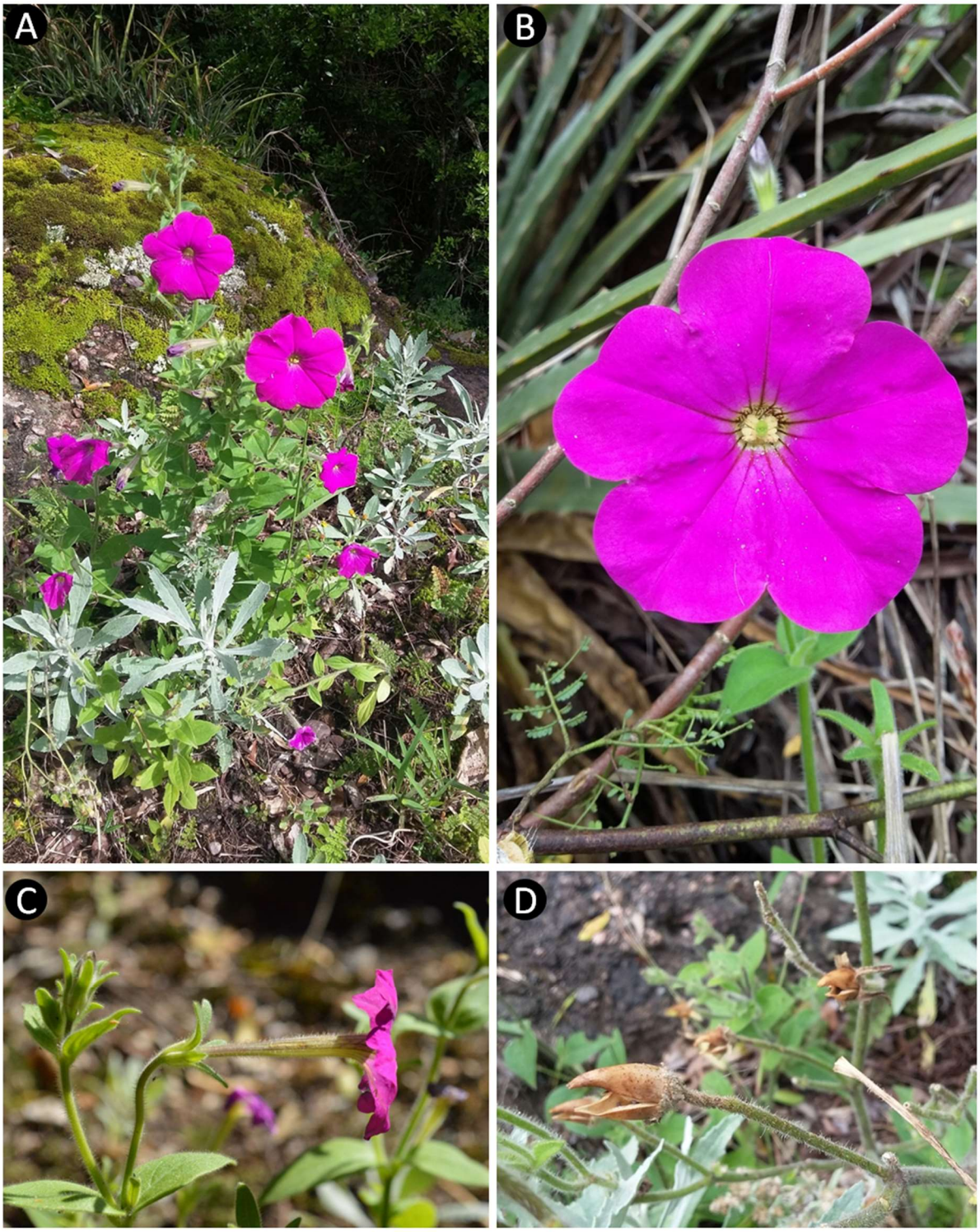

Figure S1. General aspects of morphology of *Petunia secreta*: A, General view of *P. secreta* plant; B, Anthesis finished with anthers opening; C, Flower positioned horizontally; D, Dehiscent fruit in the process of releasing the seeds.

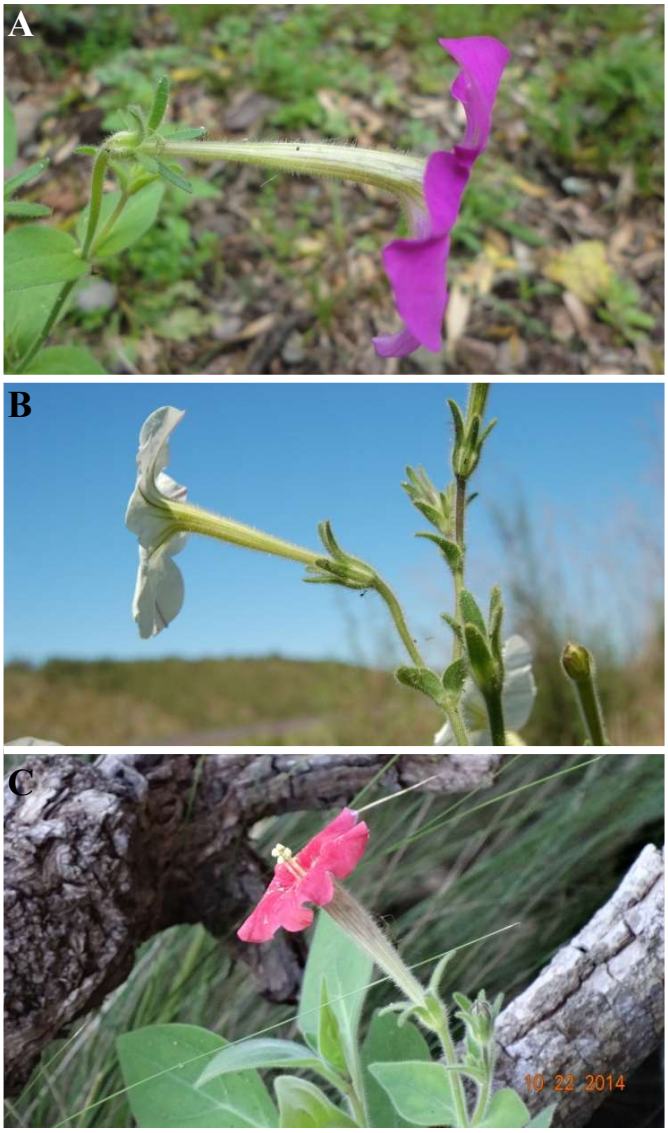

Figure S2. Corolla morphology polymorphism in the long corolla tube group of *Petunia*. A, *P. secreta*; B, *P. axillaris*; C, *P. exserta*.

Table S1. Voucher information

Species	Herbarium	Collection site	Voucher
P. secreta	BHCB *	30º54'60" S 53º55'02" W	BHCB76025
P. axillaris	ICN †	30º89'63" S 53º42'08" W	ICN185145
P. exserta	ICN †	30º83'69" S 53º50'41" W	ICN185146
P. integrifolia	ICN †	30º51'22" S 53º49'14" W	ICN181349

*Universidade Federal de Minas Gerais, Belo Horizonte, MG, Brazil; † Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil.

Table S2. Biological sources for different analyses and comparisons

Analyzes	Species	Sources
Nectar evaluation	*P. secreta*	Collected in nature; Pedra do Segredo, Caçapava do Sul, Rio Grande do Sul, Brazil.
UV light response	*P. axillaris* ssp. *axillaris* N	Rostock Botanical Garden (Germany)
	P. inflata S6	R. Koes (University of Amsterdam, the Netherlands)
	P. exserta	R. J. Griesbach (Beltsville, USA)
Pigments estimate	*P. axillaris* ssp. *axillaris*	Collected in nature; Minas do Camaquã, Caçapava do Sul, Rio Grande do Sul, Brazil.
	P. exserta	Collected in nature; Minas do Camaquã, Caçapava do Sul, Rio Grande do Sul, Brazil.
	P. secreta	Collected in nature; Pedra do Segredo, Caçapava do Sul, Rio Grande do Sul, Brazil.

Table S3. Nectar volume and sugar concentration per individual of *Petunia secreta* from Pedra do Segredo.

Individual code	Flower number	Nectar volume (µl)	Sugar concentration (%)
59A	1	4	16
59A	2	8	22
59A	3	4	23
59A	4	8	19
59A	5	4	21
59B	1	20	25
59B	2	10	25
59B	3	12	22
59B	4	10	24
59B	5	12	26
59C	1	9	23
59C	2	15	20
59C	3	4	20
59C	4	6	21
59C	5	4	20
59D	1	10	24
59D	2	8	20
59D	3	6	21
59D	4	8	21
59D	5	4	23
Mean		**8**	**21.5**

Movie S1. Unidentified bee species collecting a petal piece in *Petunia secreta*.

Movie S2. *Pseudagapostemon* sp.: The effective pollinator of *Petunia secreta*.

CAPÍTULO III

Manuscrito em preparação

Estudo preliminar de compostos aromáticos no pólen de quatro espécies de *Petunia.*

Resumo

Sinais aromáticos são característicos de flores polinizadas por insetos e estão diretamente envolvidos na atração e orientação do polinizador para os órgãos reprodutivos. O presente estudo fornece informações sobre componentes específicos dos compostos voláteis de pólen de quatro espécies de *Petunia* com diferentes traços florais e polinizadores. O aroma foi extraído utilizando a técnica de micro extração em fase sólida no modo "headspace" (HS-SPME). Os compostos foram determinados através de cromatografia gasosa acoplada a espectrometria de massa (GC/MS). Foram utilizadas as espécies *P. integrifolia* (polinizada por abelha), *P. axillaris* (polinizada por mariposa), *P. exserta* (polinizada por beija-flor) e duas linhagens de *P. secreta* (polinizada por abelha). Ao todo 77 compostos foram identificados. Os resultados mostraram 21 compostos voláteis em *P. integrifolia*, 38 em *P. exserta*, 37 em *P. axillaris*, 26 em *P. secreta* e 13 em *P. secreta-BR*. Os hidrocarbonetos alifáticos e seus derivados oxigenados foram a classe química que mais contribuiu para a composição dos aromas (61%), seguido de benzenóides (19%) e terpenos (8%). Os resultados mostraram diferenças na composição química dos voláteis entre esses cinco aromas de pólen que são discutidos em relação à biologia da polinização dessas espécies e que podem ser utilizados para interpretar seu papel potencial para a interação planta-polinizador.

Palavras-chave: *Petunia*, polinização, pólen, aroma, voláteis, HS-SPME, GC/MS.

INTRODUÇÃO

Os sinais visuais e os aromas florais são importantes na atração de polinizadores (Fenster *et al.*, 2004), por isso a análise da fragrância floral tornou-se uma abordagem integral para a compreensão da ecologia e evolução das interações planta-animal. A fragrância floral é constituída de compostos orgânicos voláteis (COVs) que, juntamente com outras sugestões florais, estimulam a atividade polinizadora e podem contribuir para o isolamento reprodutivo pré-zigótico, promovendo forrageamento de polinizadores específicos das espécies (Raguso, 2008).

A visitação de polinizadores mediada por perfume é um componente importante da capacidade do valor adaptativo das plantas (Majetic *et al.* 2009). Por exemplo, os odores de pólen são considerados, em termos evolutivos, como os mais antigos atrativos olfatórios em

flores (Faegri & van der Pijl, 1979; Dobson & Bergström, 2000). O pólen pode orientar o polinizador até as anteras (Dobson, 1991) e servir para discriminar espécies de plantas (Dobson, 1987, 1991 e 2006). Wright & Schiestl (2009) argumentam que as sugestões de perfume são facilmente aprendidas e lembradas por polinizadores porque o aroma torna as flores mais distintas e pode promover a eficiência do forrageamento, aumentar a fidelidade à fonte (Knudsen & Tollsten, 1991) e a frequência de visitação às flores (Klatt *et al.*, 2013). As plantas precisam proteger seu pólen de insetos não polinizadores e precisam anunciá-lo como recompensa aos polinizadores, porque o aroma de pólen evoluiu para proteger tanto o gametófito masculino (Irwin *et al.*, 2014), quanto para aumentar sua dispersão por animais (Parachnowitsch & Manson, 2015). Há também alguns trabalhos que demonstram a importância dos voláteis (*pollen kit*) na detecção do pólen (ver Dobson, 1994; Dobson & Bergström, 2000). Além disso, o pólen é uma importante fonte de alimento para muitos insetos, que fornecendo proteínas e lipídios que favorecem o desenvolvimento e sobrevivência de seus polinizadores (Flamini *et al.*, 2007; Muth *et al.*, 2016).

Dado que a composição química ou a cor do pólen pode influenciar a forma como os insetos interagem com as plantas (Muth *et al.*, 2016), realizar estudos abrangentes de química floral e recompensas no pólen são necessários para entender seu papel no processo de diversificação das espécies. O gênero *Petunia* tem sido objeto de estudo como um modelo de especiação mediado pela polinização (Gübitz *et al.*, 2009). O gênero está dividido em dois clados, um clado de espécies de tubo floral curto, com pólen azul e polinizadas por abelhas, e um clado com três espécies de tubo longo, pólen amarelo e com três síndromes de polinização diferentes: *P. axillaris* (mariposa); *P. exserta* (beija-flor); *P. secreta* (abelha) (Reck-Kortmann *et al.* 2014). Devido a essa história evolutiva mediada pela polinização no gênero *Petunia*, é de especial interesse conhecer a química do aroma do pólen, uma vez que envolve taxons estreitamente relacionados que são morfologicamente diferentes, simpátricos e com sobreposição de floração. A literatura cita alguns trabalhos na identificação de COVs em *Petunia* (Verdonk *et al.*, 2003; Hoballah *et al.*, 2005; Klahre *et al.*, 2011), onde verificou-se que as espécies e subespécies de *Petunia* diferiram na qualidade dos voláteis florais emitidos (Hoballah *et al.*, 2005). Por exemplo, o buquê de *P. axillaris* é dominado por ésteres benzenoides (atraentes para mariposas), álcool benzílico e benzoato de metila (Hoballah *et al.*, 2005), enquanto as flores polinizadas por beija-flores em *P. exserta* são inodoras (Klahre *et al.*, 2011) e *P. integrifolia*, polinizada por abelhas do gênero *Callonychium*, libera, quase exclusivamente, benzaldeídos (Hoballah *et al.*, 2005).

Vários estudos investigaram o significado funcional da morfologia ou cor da flor para a atração de polinizadores em espécies estreitamente relacionadas de *Petunia*. No entanto, o papel potencial das emissões de voláteis do pólen e suas diferenças entre as espécies de *Petunia* ainda não foram relatados. De acordo com as síndromes de polinização das espécies de *Petunia*, a variação da fragrância em espécies polinizadas por abelhas deve ser muito baixa. Já na espécie polinizada por mariposas, esta deve ser atribuída a um conjunto de compostos, que deve ter sido perdida na espécie polinizada por beija-flores. Nesse trabalho, buscou-se determinar se existem voláteis no pólen das espécies de *Petunia* e se há diferenças nos voláteis emitidos em cada uma delas, contribuindo assim, de forma preliminar, para o entendimento do papel dos diferentes compostos em um contexto de biologia da polinização.

MATERIAIS E MÉTODOS

Espécies utilizadas

Amostras de pólen de espécies de *Petunia* foram coletadas em populações naturais durante o período de floração no mês de novembro em 2015 a partir de 10 flores de 10 indivíduos diferentes de cada espécie. As espécies escolhidas para análise qualitativa dos compostos voláteis de pólen foram: *P. integrifolia* (HooK.) Schinz & Thell. subsp. *integrifolia,* cujo pólen apresenta coloração azul e é polinizada por abelha do gênero *Callonychium*; *P. axillaris* (Lam.) Britton, Sterns & Poggenb. subsp. *axillaris,* com pólen amarelo e polinizada por mariposas do gênero *Manduca*; *P. exserta* Stehmann, com pólen amarelo e polinizada por beija-flores; e *P. secreta* Stehmann & Semir, com pólen amarelo e polinizada por abelha do gênero *Pseudagapostemun*. Todas as espécies ocorrem na região do município de Caçapava do Sul (RS, Brasil) (Tabela 1). Da espécie *P. secreta* foram coletadas amostras de pólen das duas linhagens evolutivas descritas para a espécies (Turchetto *et al.,* 2016), uma proveniente da região da Pedra do Segredo e a outra encontrada ao longo da rodovia BR290; as amostras de *P. exserta* e *P. axillaris* foram coletadas na localidade de Minas do Camaquã, onde ocorrem em simpatria; e os exemplares de *P. integrifolia* foram coletados ao longo da rodovia BR290, mas distantes de *P. secreta* (Figura 1).

Coleta das amostras

Para cada espécie foram coletadas amostras de pólen fresco de 10 flores (junto com as anteras) em frascos de vidro e selados hermeticamente. Nas espécies *P. integrifolia* e *P. secreta* foram realizadas também amostragens com 30 flores a fim de verificar se o aumento na quantidade de material alteraria a detecção de compostos. Como se trata de uma análise qualitativa, os compostos obtidos nas duas leituras dessas duas espécies foram somados na listagem final. O pólen foi coletado uma hora após abertura das anteras, por volta das 11h da manhã.

Análises químicas

Os compostos voláteis foram extraídos e determinados através da técnica de micro extração em fase sólida no modo "headspace" (HS-SPME) e cromatografia gasosa acoplada a detector de espectrometria de massa (GC/MS). A extração dos compostos voláteis foi realizada com dispositivo Supelco SPME revestida com fibra polidimetilsiloxano/divinilbenzeno (PDMS/DVB, 100 μm). Os frascos selados foram mantidos em bloco de aquecimento, à temperatura constante de 55 ºC. Depois de atingido o equilíbrio de temperatura nos frascos (ca. cinco minutos), com a fibra retraída na agulha, o septo foi perfurado e a fibra foi exposta ao "headspace" da amostra. Após 30 min de extração, a fibra foi inserida diretamente no injetor do GC-MS para dessorção térmica dos compostos voláteis e transferência para a coluna cromatográfica. Para determinar os compostos voláteis foi utilizado cromatógrafo gasoso Shimadzu acoplado diretamente a um espectrômetro de massa modelo QP2010 (Kyoto, Japan). Os compostos foram desorvados termicamente a partir da fibra de SPME com temperatura inicial de 40º, onde permaneceu por 2 minutos, logo após passou por uma taxa de aquecimento de 8ºC/min até chegar a temperatura de 250ºC, que foi mantida durante 5 minutos. Tempo total de corrida durou 30 minutos. Os dados gerados foram analisados utilizando o software MSD Chemstation acoplado com a biblioteca de espectros de massa NIST. Os constituintes dos aromas foram descritos de acordo com a sua origem biossintética inferida a partir de Knudsen *et al.* (1993, 2006).

RESULTADOS

Classe química de constituintes de aroma de pólen

Na análise GC – MS, 77 compostos voláteis foram identificados (Tabela 2) considerando toda a amostragem. A fragrância dos pólens das espécies foi dominada por compostos hidrocarbonetos alifáticos e seus derivados oxigenados, sendo a classe química que mais contribuiu para a composição dos aromas (61%), seguida do benzeno (19%) (Tabela 2). As classes químicas alifáticas (63% em *P. integrifolia*; 70% em *P. axillaris*; 63% em *P. exserta*; 70% em *P. secreta*; e 73% em *P. secreta* BR) e benzenoide (19%; 27%; 11%; 30%, 23% respectivamente) foram detectadas em todas as espécies e representaram as principais classes químicas dos constituintes voláteis. Além disso, os compostos terpênicos estavam também presentes no aroma, mas em menor quantidade (15% em *P. integrifolia*; 5% em *P. exserta*; 2% em *P. secreta*; 4% em *P. secreta*BR; não estando presentes em *P. axillaris*). Os compostos carbocíclicos e heterocíclicos apareceram em duas espécies, *P. axillaris* (3%) e *P. exserta* (11%). Apenas um composto lipídico foi detectado, o (5Z) -Octa-1,5-dien-3-ol volátil que apareceu em amostras da espécie *P. integrifolia*.

Tipo de compostos orgânicos voláteis em pólen de quatro espécies de *Petunia*

A identificação dos voláteis revelou que cada espécie tem o seu perfil aromático de pólen específico, representando uma mistura única de compostos. O perfil de emissão das espécies tem compostos compartilhados, mas também muitos compostos únicos para cada espécie. *P. axillaris* e *P. secreta* são as espécies que mais se assemelharam, compartilhando um grande número de compostos (Tabela 2). Já as espécies *P. exserta* e *P. integrifolia* foram as que mais têm compostos exclusivos, formando um aroma muito particular (Tabela 2). O presente estudo indicou uma estreita relação entre *P. axillaris* e *P. secreta*, mas uma grande variação entre as linhagens de *P. secreta* foi encontrada. Entre os 77 compostos ativos, seis foram compartilhados entre todas as espécies: quatro compostos alifáticos (álcool isoamílico, acetoína, 2,3-butanodiol, octanol) e dois benzenóides (benzenoetanol e ftalato de isobutil). Esses compostos apareceram em diferentes proporções em cada espécie (Tabela 2), mas pode-se destacar o álcool isoamílico, que apresentou maior concentração em *P. integrifolia*

(11,66%) e *P. secreta* (7,98%). Outro composto que se destacou foi o 2,3-butanodiol, com 21,03% em *P. secreta*. No total 56 compostos foram exclusivos: 16 em *P. integrifolia*, 11 em *P. axillaris*, 23 em *P. exserta* e seis em *P. secreta* (Tabela 2).

Discussão

Este é o primeiro estudo que analisa o aroma exclusivamente do pólen em espécies de *Petunia* e foi demonstrado que as quatro espécies diferiram consideravelmente na emissão do aroma do pólen. Esta diferenciação pode ter evoluído sob seleção para a atração de séries diferentes de polinizadores ou mesmo como uma forma de proteção contra a pilhagem. Por isso, apresentamos a hipótese de que os perfis de aromas únicos de cada espécie sugerem que estas plantas podem usar diferentes "estratégias" químicas para atrair polinizadores ou evitar outros visitantes florais. Por exemplo, a emissão de terpenóides tem sido tradicionalmente associada a funções químicas de defesa (Dudareva & Pichersky, 2006; Junker & Blüthgen, 2010) e a emissão de alifáticos pode ser associada à dissuasão da herbivoria (Pichersky & Gershenzon, 2002). A maioria dos compostos do aroma identificados no presente estudo são conhecidos como componentes comuns em buquês de flores de angiospermas (Knudsen *et al.* 1993), mas os resultados mostram uma composição diferente do que é relatado pela literatura em *Petunia* (Verdonk *et al.*, 2003). Os compostos dioxigenados que estão presentes no aroma de pólen (butano-2,3-diol e acetoin) também são raros em aromas florais (Knudsen *et al.*, 1993) e foram identificados em poucas espécies.

Embora o objetivo de confirmar a presença de compostos aromáticos no pólen de espécies de *Petunia* tenha sido alcançado e uma ampla lista de compostos obtida, novos métodos de coleta e ampliação de amostragem serão realizados. Uma vez que o número e o tipo de compostos emitidos dependem do genótipo, variedades ou populações de plantas analisadas (Jakobsen & Olsen, 1994) e também das condições ambientais como a intensidade da luz e a temperatura (Kim *et al.*, 2001; Wright *et al*. 2005), novas análises deverão ser conduzidas de forma a que uma descrição mais acurada seja possível. A título de tornar significativos os resultados obtidos para comparar as espécies, deverá ser realizada nova incursão na primavera de 2017 e os dados reunidos a estes já obtidos para então serem enviados à publicação.

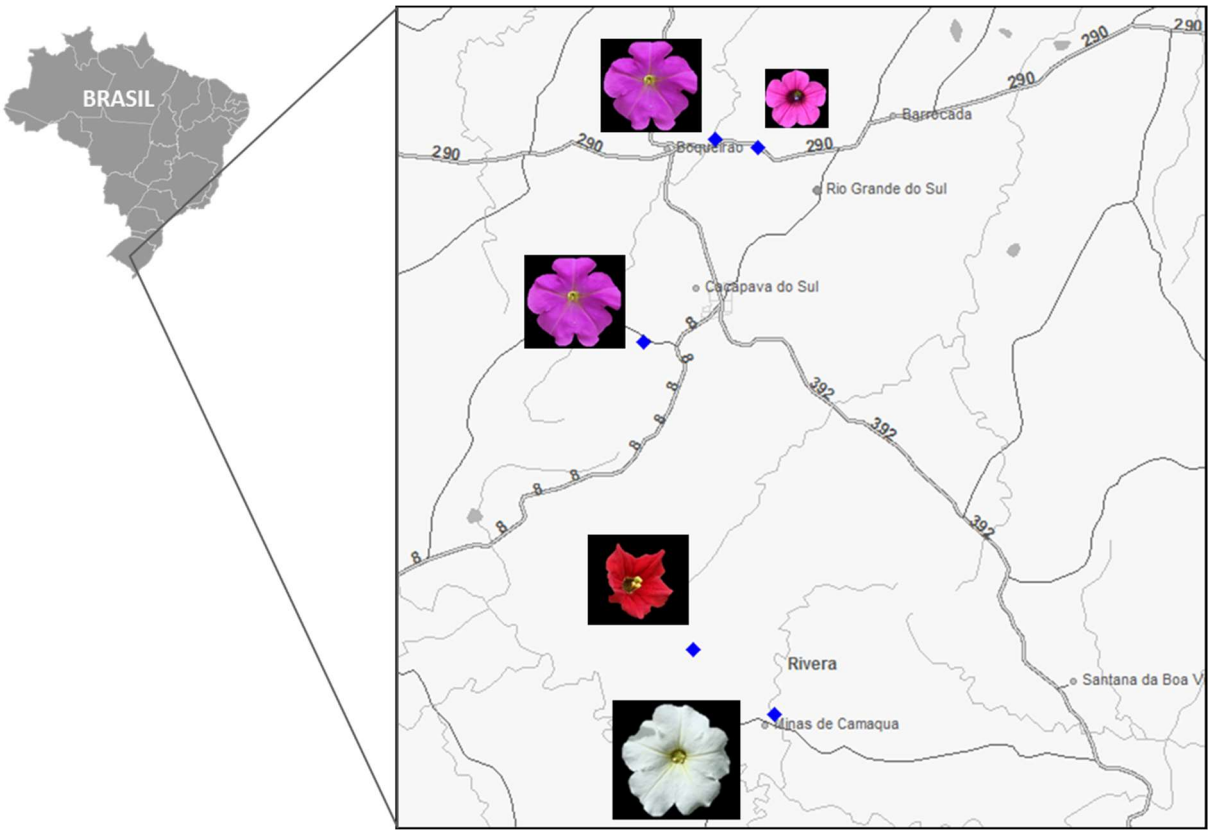

Figura 1. Mapa com os pontos de coletas realizadas na região de Caçapava do Sul para cada espécie utilizada.

Tabela 1. Informações das coordenadas geográficas do local onde foram coletadas as amostras de pólens das espécies.

Espécie	Localização	Coordenadas
P. secreta	Caçapava do Sul, Pedra do Segredo	30º32'09.56''S 53º33'06.82''W
P. secreta	Caçapava do Sul, BR	30º21'31.70''S 53º28'52.60''W
P. integrifolia	Caçapava do Sul, BR	30º22''19.60''S 53º25'38.50''W
P. exserta	Caçapava do Sul, Minas do Camaquã	30º50'18.00''S 53º30'38.00''W
P. axillaris	Caçapava do Sul, Minas do Camaquã	30º53'48.20''S 53º25'16.10''W

Tabela 2. *Checklist* dos compostos identificados por espécie.

Composto	Classe	Observações
Isoamyl alcohol	Aliphatic	Compostos compartilhados por
Acetoine	Aliphatic	

2,3-butanediol	Aliphatic	todas as espécies
Octanol	Aliphatic	
Benzeneethanol	Benzenoid	
Isobutyl phthalate	Benzenoid	
Methyl salicylate	Benzenoid	Exclusivos de *P. secreta*
Isoeugenol	Benzenoid	
2-propyl-1-heptanol	Aliphatic	
3,5,5-trimethyl-1-hexene	Aliphatic	
Hexanoic acid	Aliphatic	
3,4-dihydroxy-3,4-dimethyl-2,5-hexanedione	Aliphatic	
2,6-dimethylnonane	Aliphatic	Exclusivos de *P. axillaris*
3,7-dimethyldecane	Aliphatic	
Butanoic acid, 2-methylene-, methyl ester	Aliphatic	
(E)-2-hexenal	Aliphatic	
(Z)-hex-3-en-1-ol	Aliphatic	
3-methyl-1,5-pentanediol	Aliphatic	
2,6,11-trimethyldodecane	Aliphatic	
Tetradecane	Aliphatic	
Heptadecane	Aliphatic	
β,4-dimethylcyclohex-3-ene-1-ethanol	Cyclic compounds	
Benzyl nitrile	Benzenoid	
Hexanal	Aliphatic	Exclusivos de *P. exserta*
Undecane	Aliphatic	
Heptanal	Aliphatic	
6-ethyl-2-methyldecane	Aliphatic	
2-Hexenal	Aliphatic	
Octanal	Aliphatic	
Nonanal	Aliphatic	
β-butoxyethanol	Aliphatic	
Decanal	Aliphatic	
2-pyrrolidinemethanol, 1-methyl-	Nitrogenous	
2-ethyl-2-methyl-1,3-propanediol	Aliphatic	
Cyclohexanol, 5-methyl-2-(1-methylethyl)-, (1α,2β,5α)- Menthol	Cyclic compounds	
Nonanol	Aliphatic	
α-Terpineol	Cyclic compounds	
D-carvone	Cyclic compounds	
Azulene	Cyclic compounds	
Nerylacetone	Irregular terpenes	
Propanoic acid, 2-methyl-, 1-(1,1-dimethylethyl)-2-methyl-1,3-propanediyl ester	Aliphatic	

Propanoic acid, 2-methyl-, 2,2-dimethyl-1-(2-hydroxy-1-methylethyl)propyl ester	Aliphatic	
2-ethylhexanoic acid	Aliphatic	
Octanoic acid	Aliphatic	
3-amino-5-tert-butylpyrazole	Nitrogenous	
1,6-dioxacyclododecane-7,12-dione	Carbocyclic	
cis-caryophyllene	Sesquiterpenes	Exclusivos de *P. integrifolia*
Neoalloocimene	Monoterpeno	
p-octyloxybenzoic acid	Benzenoid	
Dinonyl phthalate	Benzenoid	
β-phenylethyl benzoate	Benzenoid	
Limonene	Monoterpeno	
5-methyl-5-propylnonane	Aliphatic	
3-octanone	Aliphatic	
Octadecane	Aliphatic	
N-tetradecane	Aliphatic	
(5Z)-octa-1,5-dien-3-ol	Lipid	
Pentadecane	Aliphatic	
Camphor	Monoterpeno	
Eicosane	Aliphatic	
Tetradecanal	Aliphatic	
Hexadecanal	Aliphatic	
Hexanol	Aliphatic	*sec* + *integ*
Methyl benzoate	Benzenoid	*sec* + *axi*
Decanol	Aliphatic	*sec* + *axi* + *exs*
Methyl phenylacetate	Benzenoid	*sec* + *axi*
Benzyl Alcohol	Benzenoid	*sec* + *axi*
Dodecane	Aliphatic	*sec* + *axi*
4,6-dimethyl-dodecane	Aliphatic	*sec* + *axi*
Hexadecane	Aliphatic	*sec* + *axi* + *exs*
Methyl heptenone	Irregular terpenes	*sec* + *exs*
1-Octen-3-ol	Aliphatic	*sec* + *integ*
Acetic acid	Aliphatic	*sec* + *exs*
Methyl Salicylate	Benzenoid	*sec* + *axi*
Toluene	Benzenoid	*sec* + *exs*
Diethyl Phthalate	Benzenoid	*sec* + *exs*
Acetyleugenol	Benzenoid	*sec* + *axi*

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CAPÍTULO IV

Artigo submetido à revista *Plant Biology*

Can the mating system explain the surprisingly high genetic diversity in a rare and narrow endemic plant species?

RESEARCH PAPER

Can the mating system explain the surprisingly high genetic diversity in a rare and narrow endemic plant species?

D. M. Rodrigues[1], C. Turchetto[1], S. M. Callegari-Jacques[2], L. B. Freitas[1,*]

[1]*Laboratory of Molecular Evolution, Department of Genetics, Universidade Federal do Rio Grande do Sul, PoBox 15053, 91501-970 Porto Alegre, Brazil;* [2]*Department of Statistics, Universidade Federal do Rio Grande do Sul, PoBox 15080, 91501-970 Porto Alegre, Brazil.*

Short Title: **Reproductive biology in *Petunia* species**

Correspondence: L. B. Freitas, Laboratory of Molecular Evolution, Department of Genetics, Universidade Federal do Rio Grande do Sul, PoBox 15053, 91501-970 Porto Alegre, Brazil.
E-mail: loreta.freitas@ufrgs.br

Keywords: Breeding system; endogamy; Pampas; *Petunia*; reproductive success; seed germinability.

ABSTRACT

- The mating system is a variable trait in flowering plants, affecting several aspects of their natural biology and it is commonly considered a major driver of diversification. *Petunia secreta* is an annual, rare, bee-pollinated species that has a fragmented distribution, while maintaining a high level of genetic diversity. In this work, we addressed several questions about the species' mating system, fruit production, and seed germinability, with the aim to understand if the mating system can partially or fully explain the species' genetic diversity.
- We sampled five populations and conducted five pollination treatments in the greenhouse: autonomous apomixis and self-pollination; hand self-pollination, geitonogamy, and cross-pollination. Overall, we analyzed 40 plants, 468 flowers, and 6,500 seeds. We evaluated the germinability success, expressed as the cumulative percentage of germinating seeds.
- Only autonomous apomixis and self-pollination did not produce fruits. All flowers resulting from hand self-pollination, geitonogamy, and cross-pollination produced fruits full of seeds. Seeds were obtained in two colors, suggesting a natural polymorphism; these germinated equally. Germinability tests were more sensitive than fruit production and each population presented a different pattern of germinability for each treatment.
- Despite depending on pollinators, *P. secreta* does not seem to have any mechanisms in place to prevent endogamy, suggesting that the species is at least partially self-compatible. Based on the present results and the genetic variability previously described, the mating systems in *P. secreta* and its large effective population size explain the high genetic diversity observed in this species, while other hypotheses seem unlikely.

INTRODUCTION

The genetic structure of a plant species is largely a consequence of the gene flow within and between populations. The mating system of a species is among the most important of the various factors that can affect its patterns of seed and pollen dispersal (Ghazoul 2005). Physical changes, such as habitat degradation or fragmentation, can affect gene flow and reproduction (Aguilar *et al.* 2008), because they reduce the abundance and density of plant individuals, resulting in a decrease in population size and an increase in spatial isolation between populations (Blambert *et al.* 2016).

Plant mating systems range from obligate cross-fertilization in self-incompatible species, requiring a mate and a pollen vector, to obligate self-fertilization in self-compatible species, which is less dependent on mates and vectors. As a consequence, a species' mating system may influence its genetic diversity (Charlesworth 2006). In the context of worldwide biodiversity loss, the management of a threatened plant species requires an understanding of its life history, and of mating and mating system as key factors determining its abundance, distribution, genetic diversity, and persistence. Management efforts should therefore build on both ecological and genetic studies.

Here, we investigated the mating system of *Petunia secreta* Stehmann & Semir (Solanaceae), a rare annual, herbaceous species that is endemic to southern Brazil (Stehmann & Semir 2005). Its distribution is restricted to two disjoint locations (Fig. 1A) at the northern edge of the Pampas region. The location where the species was first described is at Pedra do Segredo (30º 32' S/53º 33' W), a sandstone tower complex at ~300-400 m altitude, which is set amidst savanna vegetation, and where the species occurs in patches of few individuals. Outside this area, only three groups of individuals have been found, all in a flat roadside area of open vegetation, ca. 20-30 km from Pedra do Segredo, in the adjacent municipality of Caçapava do Sul (30º 21' S/53º 28' W). According to the criteria adopted by the International

Union for the Conservation of Nature, *P. secreta* is classified as critically endangered (Turchetto *et al.* 2016). Within the *Petunia* phylogeny, the species belongs to the clade with long corolla tubes (Reck-Kortmann *et al.* 2014). It can be subdivided into two genetic lineages, which correspond to the species' two locations (lineage 1, from Pedra do Segredo, and lineage 2, from the roadside ~20 km away, as described above; Turchetto *et al.* 2016). Based on morphological traits (Fig. 1B) and field observations, Stehmann & Semir (2005) have described the species as bee-pollinated. This has been confirmed through field experiments (Fig. 1C) conducted at Pedra do Segredo (D.M. Rodrigues, unpublished data). Plants at the roadside location present the same morphological characteristics, suggesting that they are also bee-pollinated, but to date, their pollinator has not been investigated. Although rare, narrow endemic, and of generally small populations, the level of genetic variability in *P. secreta* is high and of ancient origin. Microsatellite analyses confirm that today's populations are descended from a stable founder population (Turchetto *et al.* 2016).

Here, we address the following questions: (1) What is the mating system of *P. secreta*? (2) Does *P. secreta* have total or partial self-incompatibility? (3) Do artificial crossings produce seeds, and how viable are these seeds? (4) Are there any constrains to crosses of plants from different populations or evolutionary lineages in *P. secreta*? The obtained results were analyzed face to the high genetic diversity previously observed in this rare species.

MATERIAL AND METHODS

Sampling

To estimate mating parameters in *P. secreta*, we sampled five populations (Table 1) at localities representing the species' main habitats (sandstone tower complex and roadside, respectively). These five populations were chosen to cover a large area at the core of the native distribution of this species, as well as both genetic lineages (Turchetto *et al.* 2016). The

distance among sampled populations ranged from ~0.2 km (BR163 and BR573) to 1.2 km (PS59 and GP62). The Pedra do Segredo and roadside assemblies are at a distance of ~22 km from each other. For the manual crosses, we collected mature seeds from five individuals in nature during the flowering season of 2014 (September-December). The seeds were then germinated in a growth chamber at 22º C in a 12-h light:12-h dark cycle. To maximize germination, the seeds were pre-treated with a 100 μM solution of gibberellic acid (GA_4; Sigma-Aldrich Co., St. Louis, MO, USA), dissolved in 1 mL DMSO (dimethyl sulfoxide; Sigma-Aldrich) and then diluted in water (Ali-Rachedi *et al.* 2004). Treatment was applied in a dark chamber at 4º C for 24 h. Germination began four days after planting, and the initial germination success was high (88.4 ± 4.6%, population mean ± SE, N = 150 seeds). Eight seedlings per population were randomly chosen for transfer to a greenhouse, where they were cultivated until bloom in accordance with the standard practice for garden petunias, for a total of 40 plants.

Mating experiments

To determine the mating system of *P. secreta*, five pollination treatments were applied in the greenhouse: (1) autonomous apomixis: buds were bagged in a mesh bag, and anthers were removed before dehiscence; (2) autonomous self-pollination: buds were bagged throughout their flowering period; (3) hand self-pollination: bagged flowers were hand pollinated with pollen from the same flower; (4) hand geitonogamy: bagged flowers were hand pollinated with pollen from a different flower from the same plant; (5) hand cross-pollination: bagged flowers were emasculated before anthesis and pollinated with pollen from plants obtained from other populations. For all experiments, bags were kept until fruit spontaneously opened. The number of fruits was recorded after three weeks, and the fruit set was estimated as the proportion of flowers setting fruits. Mature fruits were collected and their seeds kept at 4º C. Overall, we analyzed 468 flowers across all plants, populations, and pollination treatments

126 (see Table 2 for number of flowers per population per treatment). We also recorded several
127 morphological characters of the plants, including flowers, fruits, and seeds, to characterize the
128 two lineages of *P. secreta.*

129 ***Seed production and germinability***

130 For each pollination treatment, we collected the seeds of three randomly selected fruits in a
131 0.2 µL tube. The seeds were weighed, and their weight served as a representative estimate of
132 seed production. Up to 100 randomly selected seeds per fruit were germinated in a growth
133 chamber at 22º C in a 12-h light:12-h dark cycle, as described previously. The total number of
134 seeds was 6,500. Upon emergence of the seedlings, the temperature was increased to 25º C.
135 Seedlings with open cotyledons were counted every seven days for seven weeks after
136 planting. A comparison of germinability between seeds from different treatments and of
137 different colors (see results) was carried out based on 200 randomly chosen seeds covering all
138 colors and treatments.

139 ***Data analysis***

140 Seedlings were observed until the cotyledons were fully open. Germinability was expressed
141 as the cumulative percentage of seeds that had germinated by the end of the experiment. The
142 statistical evaluation of seed germination data is not straightforward (McNair *et al.* 2012).
143 Nevertheless, similar to a seed technology test, our intention here was to compare possible
144 ways of pollination to provide an estimate of new plants potentially available in nature, and
145 the chosen statistical tests were sufficient for this purpose (ISTA 1985). We used the Chi-
146 square and Kruskal-Wallis tests implemented in the WinPEPI software suite (Abramson
147 2011) and PASW/SPSS software to compare germinability across treatments and populations.
148

149 **RESULTS**

150 ***Vegetative growth and number and size of flowers***

151 During the initial developmental stages, plants from different populations and lineages
152 presented identical vegetative morphology, with a rosette arrangement for all leaves. Just
153 before bloom, the rosette-type shoots elongated in plants from evolutionary lineage 1 of *P.*
154 *secreta* (PS59 and GP62, Pedra do Segredo locality). In plants from lineage 2 (BR142,
155 BR163, and BR573, roadside locality) four or five lateral shoots emerged before the
156 elongation of the central rosette-type shoot. Plants from lineage 2 hence invested more in
157 vegetative growth than plants from lineage 1. Nevertheless, no differences in flower numbers
158 and in numbers of individual plants producing more than 20 flowers were observed between
159 populations (Fig. S1A, B). As observed in nature, all individuals were able to maintain
160 flowers and fruits simultaneously. Plants survived in the greenhouse for eleven months.

161 ***Timeline of flower opening and senescence and fruit production***

162 As observed in nature (D. M. Rodrigues, unpublished data), flowers of *P. secreta* opened
163 during the daytime and remained open for four days if not pollinated and the senescence
164 began around four days later with a change in the color of the corolla, followed by the gradual
165 wilting of the petals. Senescence starts two days after the pollination. Anther dehiscence
166 occurred simultaneously with the opening of the flower (around 30 minutes) and the anthers'
167 position was always below the stigma. No differences were observed between plants or
168 populations. The wilted corolla remained attached to the calix if not fertilized, and fell if fruits
169 were growing (Fig. S1C, D).

170 Among the five pollination treatments applied here, only autonomous apomixis and
171 autonomous self-pollination did not produce any fruits, indicating that *P. secreta* is dependent
172 on the pollinator to fructify while being, at least partially, self-compatible.

173 ***Seed quantity estimates***

174 In all plants, the fruit-stalk was erect and contained hundreds of seeds (Fig. S1E, F). For each
175 treatment, we estimated the seed content from the weight of 0.2 mL of seeds from randomly

176 selected fruits. No differences in weight were observed between pollination treatments that
177 did produce fruit (Kruskal-Wallis test; $P > 0.05$). The weight of 0.2 mL of seeds was as
178 follows (mean of three fruits ± SE): hand self-pollination, 0.193 ± 0.01 g; hand geitonogamy,
179 0.198 ± 0.01 g; hand cross-pollination, 0.188 ± 0.01 g.

180 ***Seed germinability***

181 For hand self-pollination, hand geitonogamy, and hand cross-pollination within and between
182 lineages, all tested flowers produced fruits full of seeds. The obtained seeds were of two
183 different colors, brown and grey (Fig. S1E, F). These colors were observed for all modes of
184 pollination, individuals, and populations, suggesting a natural polymorphism. Seed color
185 proportions were similar for all treatments and ranged around 50%. No correlation was
186 observed between seed color and germinability (Kruskal-Wallis test; $P > 0.6$). Considering all
187 pollination treatments and plants, a mean of 68% of seeds germinated for each color, with a
188 range of 0% to 100%.

189 Germinability tests showed greater differences between plants and pollination
190 treatments than fruit production. Each population presented a different pattern of
191 germinability in relation to pollination treatments (Fig. 2; Table 3). The only population that
192 did not show differences in germinability between pollination treatments was BR573, where
193 all capsules showed a similar mean of germinability (Fig. 2). In general, germinability indices
194 were lower in plants from PS59 and GP62 (lineage 1) than in plants from BR142, BR163, and
195 BR573 (lineage 2) (Fig. 3A). The lowest values were observed for plants from GP62 who had
196 received pollen from PS59 (Fig. 3B). In plants from PS59, GP62, and BR142, capsules
197 obtained from hand geitonogamy had a higher seed germinability than those obtained through
198 hand self-pollination (Fig. 3B, C). In plants from BR142, no differences were observed
199 between hand geitonogamy and hand outcrosses, independently of the pollen donor, whereas
200 for plants from BR163, a lower germinability was observed for capsules resulting from

outcrosses with plants from PS59 or GP62 (Fig. 3C). Independent of the mode of pollination, plants from PS59 had a higher germinability than plants from GP62 (Fig. 3B), whereas in lineage 2 (Fig. 3C), plants from BR142 had the lowest germinability (except when these plants received pollen from plants from lineage 1). All differences were statistically significant, with $P < 0.001$.

Considering all plants, populations, and treatments, the average rate of germinability was 73% (9% to 100%; Table 3). Pollination treatment results differed between populations (data not shown) and between lineages (Fig. 3A). There were no differences in germinability between pollination treatments within lineage 2, whereas the highest germinability was observed after hand geitonogamy in lineage 1.

DISCUSSION

The mode of reproduction affects several aspects of the natural biology of plants, such as the homozygosity and genetic variability of their populations, and the mating system is therefore commonly considered a major driver of lineage diversification (Barrett 2013).

Here, we investigated the mating system of *P. secreta* through a range of hand pollination experiments. *P. secreta* is an annual species that blooms from September to December, throughout the Southern Hemisphere's spring season. It has pink flowers characterized by a long and salveform corolla (i.e., a tubular-shaped corolla with a flat expanded limb) and yellow pollen (Stehmann & Semir 2005). The geographic range of the species is fragmented, with small populations at Pedra do Segredo, and slightly larger populations at a roadside locality ~20 km from Pedra do Segredo. Although all populations fluctuate substantially in size from one generation to the next, the species has a surprisingly high level of genetic diversity compared to other *Petunia* species (Turchetto *et al.* 2016).

In nature, solitary bees visit *P. secreta* (Stehmann & Semir 2005) and effectively

226 transfer pollen to the stigma (D.M. Rodrigues, unpublished data; Fig. 1C). However, to date,
227 no pollination experiments have been conducted in this species. The prevalent breeding
228 system could explain the high genetic variability in this species with a pattern of small
229 populations distributed over a fragmented area (Fig. 1A).
230 According to Charlesworth (2006), it is useful to distinguish two types of plant mating
231 systems, which can both contribute to the understanding of genetic diversity and population
232 structure in plant species: (1) "sex systems", which consider whether the plant is monoecious,
233 dioecious, or other, and (2) the mating systems of monoecious plants, including inbreeding,
234 outcrossing, and intermediate systems of crosses. *Petunia secreta* is a monoecious plant, in
235 which the stigma is located slightly above the anthers (Stehmann & Semir 2005; Fig. 1B).
236 Additionally, *P. secreta* displays several characteristics to attract bees (D.M. Rodrigues,
237 unpublished data; Fig. 1C), suggesting that the species is dependent on a pollinator. Our
238 results fully confirm this, as neither autonomous apomixis nor autonomous selfing led to the
239 production of fruit.
240 Although dependent on a pollinator, *P. secreta* does not seem to have any mechanisms
241 to prevent inbreeding since hand selfing and geitonogamy produced fruits in all studied
242 populations. In addition, the seed germinability was higher in several of these fruit than in
243 fruit produced through other modes of pollination, suggesting that *P. secreta* is, at least in
244 part, self-compatible (Figs. 2 and 3). The loss of self-incompatibility and improvement of
245 endogamy have been seen in *P. axillaris* populations occurring in sympatry with *P. exserta*
246 (Turchetto *et al.* 2015). *P. secreta* grows in the same area as *P. axillaris* and *P. exserta*, which
247 belong to the same *Petunia* clade as *P. secreta*, and as *P. integrifolia,* which is also bee-
248 pollinated.
249 Reproductive success in stressful or variable environments, in which pollination may be
250 uncertain, is a common problem for many lineages of seed plants. For outcrossing species in

disturbed habitats, pollinator limitation and a restricted partner availability are commonly encountered problems (Yin *et al.* 2016). *Petunia secreta* has a fragmented distribution across disturbed areas, where plants are generally found in small populations (Turchetto *et al.* 2016). While these attributes generally suggest a depletion of genetic diversity (Ellstrand & Elam 1993), they are not reliable predictors of genetic dynamics (Premoli *et al.* 2001), as is demonstrated by our findings in *P. secreta*. The rate of seed germinability in an endogamous setting was at least equivalent to that obtained from outcrossing (Fig. 3), suggesting a high tolerance of inbreeding in this species. This seems to enable *P. secreta* to overcome the limitations in partner availability and to keep high genetic diversity indices despite a low level of long distance gene flow.

Plant species have evolved a range of reproductive strategies to cope with unfavorable pollination conditions. For example, plants faced with severe pollinator restriction, and hence reduced possibilities for cross-pollination, can ensure reproductive success by increasing self-compatibility (Larson & Barrett 2000). In *P. secreta*, both hand selfing and geitonogamy resulted in high levels of seed germinability (Table 3).

Likewise, prolonged floral longevity can compensate for a low rate of pollinator visitation (Barrett 2003) and can be seen as an evolutionary strategy to overcome a sparse or unpredictable pollinator service (Steinacher & Wagner 2010). In line with this, a recent study showed that flowers with longer exposure times had greater chances to be visited by pollinators, as well as a higher reproductive success (Darling & Barrett 2011). Here, we observed that, in the absence of pollination, *P. secreta* maintains open and fresh flowers with a receptive stigma for four days. This is similar to the flower durability observed in nature (D. M. Rodrigues, unpublished data).

Unpredictability in pollinator and partner availability can lead to selection for a combination of self-compatibility and improved outcrossing pollination strategies (Kalisz *et*

276 *al.* 2004). This seems to be the case in *P. secreta*, since the differences in germinability were
277 greater between individual plants and populations, than between pollination treatments (Figs.
278 2 and 3).

279 In small populations, several evolutionary processes, including genetic drift, can lead to
280 a reduction in genetic variation and increase the rate of endogamy with all its negative
281 consequences to maintain the population. Mechanisms preventing self-fertilization might act
282 in very small populations. In line with this, an increase in the proportion of heterozygotes has
283 been observed in sexual species (Cole 2003). When a species is divided into a series of small
284 populations, which are so isolated from each other that dispersal rarely carries genes from one
285 population to another, genetic drift can lead to divergent allele frequencies in the isolated
286 populations, increasing levels of several diversity indicators (Ellstrand 1992). As previously
287 shown through the analysis of microsatellites, F_{ST} values are higher in *P. secreta* than in other
288 *Petunia* species with a larger geographic distribution and/or bigger populations (Turchetto *et*
289 *al.* 2016). Thus, inbreeding coupled with occasional gene flow due to pollen or seed dispersal
290 between populations could satisfactorily explain the genetic structure found in *P. secreta*.

291 Genetic diversity is important both for short-term adaptations to microhabitat
292 differentiation, and for long-term survival through evolutionary adaptation to changing
293 environments (Furches *et al.* 2013). The microhabitats occupied by *P. secreta* populations
294 show significant differences, especially in their soil substrate and associated vegetation.
295 While populations from lineage 1 grow in shallow soil directly on the rock and are
296 surrounded by xerophytes, populations from lineage 2 are found in deep and rich soil
297 surrounded by grass. Both environments are modified and suffer continuous impact from
298 human activities, such as cattle rising, agriculture, and car traffic. Among populations from
299 lineage 1, some plants grow on a relatively preserved property, while others grow on a farm.
300 The populations from lineage 2 were collected from different sides of the road; all are

301 subjected to traffic impact. Although our experiments were conducted in controlled
302 conditions, the initial seeds were obtained from their natural locations, bringing with them the
303 genes underlying their adaptions to a range of challenging environments. Since all plants were
304 cultivated under the same conditions, we disregard transient responses to the pollination
305 treatments and believe that the seed germinability is a natural response of *P. secreta* to
306 maintain genetic variability.
307 The high degree of population scattering in *P. secreta* may be explained most probably
308 by the species' herkogamic flowers than through some molecular mechanisms of self-
309 incompatibility (see results on hand self-pollination Fig. 3). These morphological traits may
310 have been responsible for maintaining a high level of genetic diversity by reinforcing
311 outcrossing strategies in the face of potential inbreeding.
312 Various factors affecting the mating system, including the mode of pollination,
313 pollinator viability, and the presence of molecular or structural self-incompatibility systems
314 (Karoon *et al.* 2012). Moreover, when pollinators move only over short distances, as in the
315 case of the solitary bees found to visit *P. secreta* (Stehmann & Semir 2005; Fig. 1C), and
316 when pollen carryover is limited, as it might happen in the small populations of *P. secreta*
317 (Turchetto *et al.* 2016), a large fraction of the pollen deposited on a stigma might come from
318 closely related individuals (Mitchell *et al.* 2009). Alternatively, it might constitute a mix of
319 self-pollen, pollen of relatives, and pollen from distant donors. If all pollen, independent of its
320 origin, has the same ability to fertilize and develop viable seeds, as we observed here for all
321 pollination treatments (Figs. 2 and 3), high diversity levels would be obtained, both for the
322 population and for the species in general.
323 There is a plethora of studies comparing the genetic variation of rare plants to those
324 with a wide distribution. Many of these indicate a strong correlation between genetic diversity
325 and geographic range, since species with a larger distribution would present higher levels of

variability (Binks *et al.* 2015). However, like *P. secreta*, there are several rare species that have been found to exhibit equal or greater genetic diversity than their widespread relatives (Turchetto *et al.* 2016). Clearly, the genetic dynamics of a species should not be predicted based on its geographical range alone. Indeed, there are a myriad of factors that can influence the levels of diversity within and between populations, including the species' mating system (Coppi *et al.* 2014) and large effective population sizes, a common explanation for the maintenance of diversity in rare species (Ellstrand & Elam 1993).

Based on the present results and on the genetic microsatellite variability described in a previous paper (Turchetto *et al.* 2016), we conclude that the mating systems in place in *P. secreta*, coupled with a large effective population size, may be responsible for the high diversity observed in this species.

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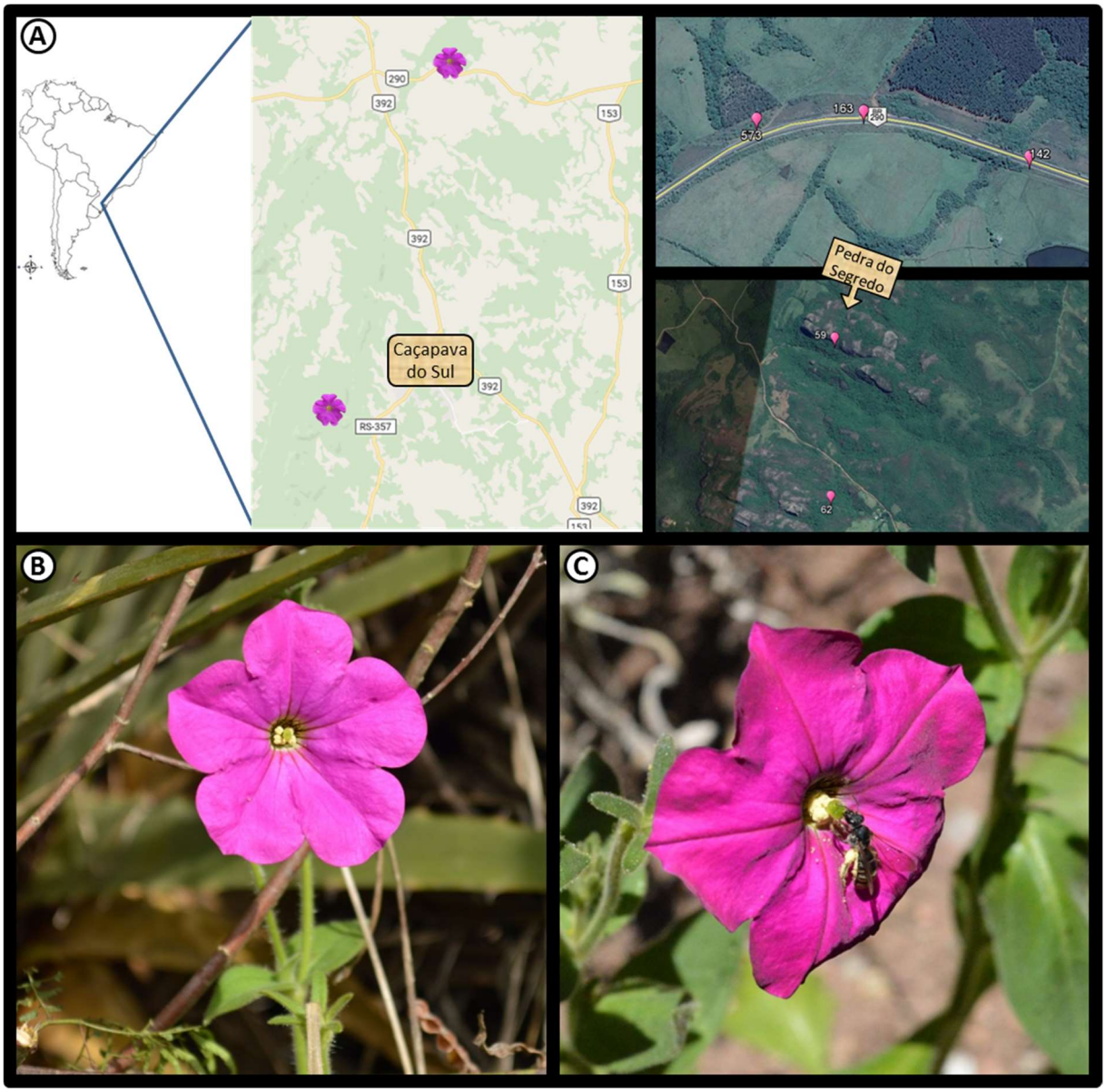

Figure 1. Distribution of *Petunia secreta* and overview over its morphology. (A) Studied populations (Caçapava do Sul municipality, Brazil); (B) General view of the plant; (C) *Pseudagapostemon* sp. collecting pollen from *Petunia secreta* in Pedra do Segredo.

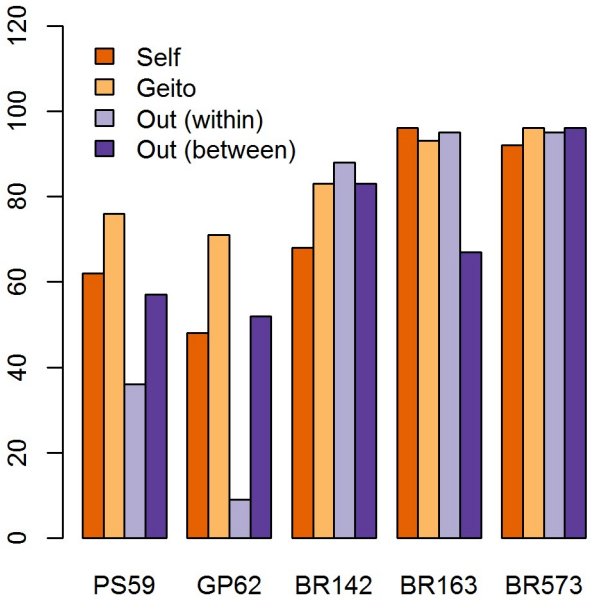

Figure 2. Comparison of germinability between populations and pollination treatments in *Petunia secreta*. Each bar represents the mean of seed germinability estimated as the proportion of seedlings with open cotyledons per treatment per population; self – hand pollination using pollen of the same flower; geito – hand pollination using pollen of a different flower from the same plant; out (within) – outcrossing using pollen of a different flower from the same evolutionary lineage; out (between) – outcrossing using pollen of a flower from a different evolutionary lineage.

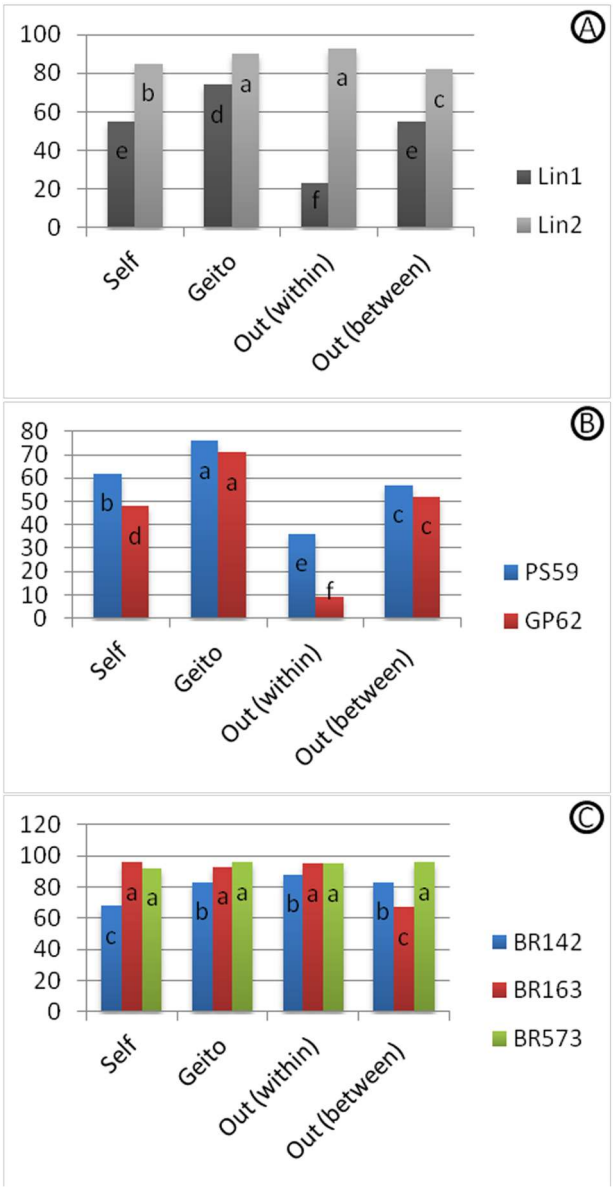

Figure 3. Comparison of the germinability after different pollination treatments in different populations and evolutionary lineages of *Petunia secreta*. (A) Comparison between the two evolutionary lineages; (B) Comparison between two populations from Pedra do Segredo locality (lineage 1); (C) Comparison between three roadside populations (lineage 2). Different letters indicate statistical significance ($P < 0.001$).

SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article:

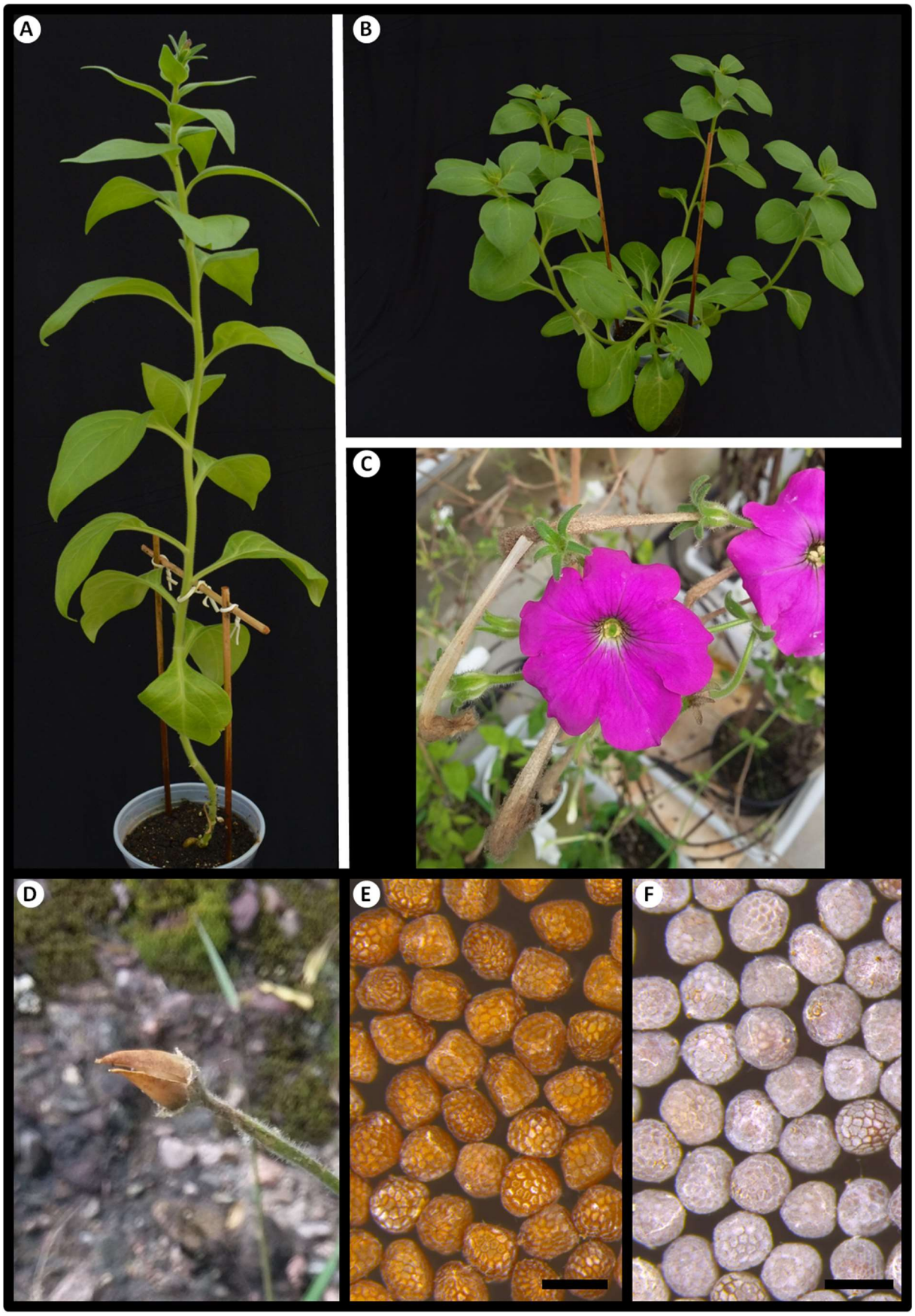

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Figure S1. Morphological traits of *Petunia secreta*: (A) General appearance of a typical individual from Pedra do Segredo; (B) General appearance of a typical individual from a roadside population; (C) Frontal view of a flower with wilted corollas attached to the calix after pollination; (D) Mature and open fruit; (E) brown and (F) grey seeds under the stereomicroscope. Bars = 2 mm.

Table 1. Sampling information for plants collected in nature.

Population	Location	Evolutionary lineage*	Coordinates
PS59	Pedra do Segredo	1	30°32'09.56"S 53°33'06.82"W
GP62	Pedra do Segredo	1	30°32'45.90"S 53°33'00.97"W
BR142	Roadside	2	30°21'32.79"S 53°28'33.73"W
BR163	Roadside	2	30°21'30.80"S 53°28'45.38"W
BR573	Roadside	2	30°21'31.70"S 53°28'52.60"W

*According Turchetto *et al.* (2016)

Table 2. Number of flowers per pollination treatment used in *Petunia secreta*

Treatment	PS59	GP62	BR142	BR163	BR573
Autonomous apomixes	16	11	21	15	14
Autonomous self-pollination	19	13	26	15	17
Hand self-pollination	18	12	20	15	17
Hand geitonogamy	15	9	15	14	16
Hand cross-pollination	25	13	23	49	40

458 **Table 3.** Seed germinability expressed as percentage of seedlings with open cotyledons per population per treatment in *Petunia*

459 *secreta* considering eight plants per population.

460

	PS59			GP62			BR142			BR163			BR573		
	Mean[1]	Min	Max	Mean	Min	Max	Mean	Min	Max	Mean	Min	Max	Mean	Min	Max
Self[2]	45	30	87	48	10	83	68	42	94	96	95	96	92	88	95
Geito[3]	59	21	100	30	9	98	83	76	89	93	91	94	96	95	96
Out (within)[4]	36	9	100	9	10	9	87	78	93	95	92	98	95	93	97
Out (between)[5]	57	9	95	48	26	92	83	56	99	67	22	92	96	92	100

461 [1] % estimates were based on overall 6.500 seeds cultivated in growth chamber and controlled conditions during seven weeks; [2]hand pollination using pollen from the same flower;
462 [3]hand pollination using pollen from different flower from the same individual; [4]hand outcrossing using pollen from individuals from the same evolutionary lineage; [5]hand
463 outcrossing using pollen from individuals from different evolutionary lineage; Mean – considering at least 100 seeds per plant and eight plants per population; Min – minimum
464 number of seedlings; Max – maximum number of seedlings.
465

CAPÍTULO V

Manuscrito em preparação para ser submetido à revista *Annals of Botany*

Diverse yet endangered: gene flow and genetic diversity in a narrow endemic plant species.

Original article

Diverse yet endangered: gene flow and genetic diversity in a narrow endemic plant species

Daniele M. Rodrigues[1], Caroline Turchetto[1], Loreta B. Freitas[1,*]

[1]Laboratory of Molecular Evolution, Department of Genetics, Universidade Federal do Rio Grande do Sul, PoBox 15053, Porto Alegre, Brazil

[2]Author for correspondence (e-mail: loreta.freitas@ufrgs.br)

Running title: Gene flow in a rare species of *Petunia*.

[*]*For correspondence*. E-mail loreta.freitas@ufrgs.br

Abstract

Background and Aims The mating system is an important component to understand the reproductive success e persistence of species. Indeed, the identification of the mode of pollen dispersal is central to the conservation of endangered species. The *Petunia* genus has recent diversification history and more than half of species present small geographic distribution. As a matter of fact, *P. secreta* is a rare and endemic species of genus from a particular rock formation in southern Brazil. Although narrow endemics have historically been hypothesized to have low levels of genetic diversity, *P. secreta* present a high genetic variability, either when compared to *Petunia* species widely or restrict distribution. The main aim of this study was to estimate parameters concerning the mating system of *P. secreta* to evaluate the role of gene flow by pollen can contribute to the maintenance of the genetic diversity in this species.

Methods During one bloom season we 46 adult individuals from seven natural populations in the original geographic distribution of *P. secreta*. We randomly sampled eleven individuals in nature as mother-plants collected fruits from open-pollinated flowers to germinate the seeds in greenhouse. We obtained 125 seedlings and determined the genetic profile of the 171 individuals (adults and offspring) using nine polymorphic microsatellite loci. Analysis of diversity, genetic structuring, and paternity were estimated.

Key Results No geographical structure was observed in adults individuals of *P. secreta,* with populations presenting different genetic components among individuals (*K=4*; structure analysis). Comparison of the allelic frequencies among adult individuals and between mother plants and their progenies allowed attributing 100% of paternity inside the sampled area and no immigrant pollen was observed. Our results suggest that *P. secreta* present a mixed mating system with predominant self-fertilization, with few pollinator events in each mother plant. Moreover, all cross pollination events were determined as occurring at 0 m of distance (inside populations). Inbreeding depression value was high suggesting that fitness of *P. secreta*

progeny can be affected by inbreeding depression. Inbreeding also was observed in adult plants as the positive F_{is} values.

Conclusions These results suggested that *P. secreta* preferably do self-pollination possibly influenced by the pollinator's behaviour. We discuss about the genetic diversity in this species that could be attributed to an intra-population spatial genetic structure in each collection site perhaps influenced by temporal gene flow. However, inbreeding depression was identified and this may have implications in survival of species. These results should be taken into account to propose strategies for *in situ* species' conservation.

Key words: Gene flow; microsatellites; Pampa; *Petunia*; pollination; pollen dispersal Solanaceae.

INTRODUCTION

One of the criteria adopted by International Union for the Conservation of Nature (IUCN) to classify a species as threatened of extinction is its restricted geographic distribution (http://www.iucnredlist.org/). The geographic distribution can be related with genetic variability. For example, reduced genetic variability is expected in species with small population sizes or restricted geographic distribution (Hamrick and Godt, 1989). Insufficient variability would lead species to be more vulnerable to extinction under selection pressure (Sheth and Angert, 2014). Rare and endemic species are highly priority for conservation strategies because they are more susceptible to extinction just because their restricted geographic distribution (Isik, 2011). Knowledge on genetic diversity and gene flow within a species and how these parameters are distributed among populations has long been thought to be helpful to draw conservation programs and management of protected species, and is also fundamental to the understanding the evolutionary processes that have shaped the species (Hamrick and Godt, 1996).

The processes of dispersion of species are a causal factor in the dynamics of populations, communities, and ecosystems (García *et al.*, 2017), as well as the gene flow. The systems of crossing and dispersal of alleles through pollen and seeds determine how the genetic information will be transferred across generations and how the diversity will be distributed among individuals within a species (Ghazoul, 2005). Therefore, identifying the reproductive mode and understanding the pollen dispersal of a species are fundamental to propose strategies for species' conservation (Adams and Burczyk, 2000).

The genus *Petunia* Juss. has 14 species and most of them are endemic to restricted areas (Stehmann *et al.*, 2009). *Petunia secreta* Stehmann & Semir is a rare and endemic species found only in a rock formation called Pedra do Segredo, Caçapava do Sul municipality, in the Brazilian state Rio Grande do Sul. This bee pollinated species displays a

73 long and pink corolla tube, yellow pollen and grows in the open areas from a conglomerate
74 sandstone at ~ 300–400 m high (Stehmann *et al.*, 2005). Only few individuals composed the
75 populations of *P. secreta* and this number varies with each generation (Turchetto *et al.,* 2016).
76 The sites are ca. 200 m distant one from each other.
77 In the Pedra do Segredo locality, there is a very small preservation area called Pedra
78 do Segredo Municipal Park, the unique formal place for the maintenance of the species *P.*
79 *secreta*. The neighbourhood of this park is formed by private properties that make use of fire
80 to clean fields and raise cattle and goats. All these activities put *P. secreta* in risk, emphasizing
81 the importance of the park for the maintenance of this species.
82 Contrary to that was expected for a rare species, Turchetto *et al.* (2016) found a
83 relatively high levels of genetic diversity to *P. secreta* when compared to other *Petunia*
84 species with wide or narrow distribution. These findings corroborated other works
85 demonstrating that may exist other genetic and ecological scenarios for rare and endemic
86 species (see Fernández-Mazuecos *et al.* 2014; Jiménez-Mejías *et al.* 2015; Fernández-
87 Mazuecos *et al.* 2016; Forrest *et al.* 2017).
88 The gene flow is one of the evolutionary forces that directly affect the change of allele
89 frequencies and can influence on genetic variability from generation to generation (Slatkin,
90 1985; Ellstrand, 2014). Therefore, our main objective was to analyse the extension of gene
91 flow by pollen and discuss about their role in maintenance the genetic variability of the
92 species. Also we aimed to evaluate parameters concerning mating system to observe the risk
93 of *P. secreta.*
94
95 **MATERIALS AND METHODS**
96 ***Study site and sampling***
97 In the spring of 2014 we analysed 46 individuals of *Petunia secreta* from the Pedra do

Segredo locality. These individuals were distributed in seven different populations, two of them located in the Pedra do Segredo Municipal Park, one population from a private property close to the park, and four populations within a private property called Galpão de Pedra in front of the park (Fig.1). The geographical coordinates of the populations were obtained through the Global Positioning System (GPS). The number of adult individuals in each population ranged from one to 16 (Table 1).

All individuals (46) had leaves collected, dried on silica and powdered to DNA extraction. Eleven individuals were randomly chosen as mother plants (Fig. 1 and Table 1) to covering the entire occurrence area in Pedra do Segredo. We collected fruit from each mother plant and germinated the seeds in a growth chamber with temperature and luminosity controlled. All seeds were pre-treated to break possible physiological dormancy associated with seasonal weather changes. The pre-treated consisted in submerging seeds in a 100-µM solution of gibberellic acid (GA_4; Sigma-Aldrich Co., St. Louis, MO, USA) dissolved in 1 mL DMSO (dimethyl sulfoxide; Sigma-Aldrich) and then diluted in water (Ali-Rachedi *et al.* 2004) for 24 h in the dark at 4 º C before planting. We were able to germinate 4 – 15 seeds per mother plant generating a total of 125 progenies. The genomic DNA was isolated from overall 171 individuals (46 adults and 125 progenies) using the CTAB (cetyltrimethy lammonium bromide) protocol described by Roy *et al.* (1992).

Characterization of microsatellite loci

The adults and progenies were genotyped using nine informative microsatellite loci named PM184, PM88, PM177, PM167, PM183, PM188, PM173, PM195, and PM92 previously described to *P. hybrida* (Bossolini *et al.*, 2011). The polymerase chain reactions (PCRs) were conducted according to protocol established in Turchetto *et al.* (2015). These markers were successful used to describe the genetic diversity in *P. secreta* and sisters species (Turchetto *et*

123 *al.*, 2016). The amplified DNA was denatured and size-fractionated using capillary
124 electrophoresis on an Applied Biosystems (Foster, CA, USA) with a LIZ (500) molecular size
125 standard (Applied Biosystems). The GeneMarker 1.97 software (Softgenetics LLC, State
126 College, PA) was used to determine the alleles. Additionally, all alleles were visually verified
127 and scored.

128 To estimate genotyping errors due to stutter bands, allele dropout, and null alleles, we
129 used Micro-Checker software (Oosterhout *et al.*, 2004; http://www.microchecker.hull.ac.uk/).
130 To obtain the number of alleles per locus and the observed (H_O) and expected (H_E)
131 heterozygosity under Hardy–Weinberg equilibrium (after Bonferroni's correction), we used
132 Arlequin 3.5.1.2 software (Excoffier and Lischer, 2010). To obtained linkage equilibrium for
133 all loci (Goudet *et al.*, 1996) with Bonferroni's correction and inbreeding coefficient (F_{IS}) we
134 used Fstat 2.9.3.2 software (Goudet, 2002; http://www2.unil.ch/popgen/softwares/fstat.htm).
135 Genetic identity (I; Chakravaratt and Li, 1983) and paternity exclusion (*Q*; Weir, 1996)
136 probabilities were estimated for each locus, and paternity exclusion {*QC* 1/4 1[*P*(1*Q i*)]} and
137 genetic identity (*IC* 1/4 *PI i*) combined probabilities were estimated for the overall loci using
138 the Identity 1.0 software (Wagner and Sefc, 1999). All these analyses and estimates were
139 performed considering all 46 adult individuals.
140
141 ***Population structure***
142 We implemented the Analysis of Molecular Variance (AMOVA; Excoffier *et al.,* 1992) in
143 Arlequin among the seven populations and between Pedra do Segredo and Galpão de Pedra to
144 quantify the genetic variance among the different localities. The F_{ST} par a par was run in
145 Arlequin to evaluate the levels of differentiation between populations with 10 000
146 permutations to assess significance.
147 The population structure of 46 adults individuals was analysed using coalescent

148 approach implemented in Structure 2.3 software (Pritchard *et al*., 2000) using as parameters:
149 admixture model assuming independent allele frequencies without a priori information about
150 population assignments of individuals; burn-in of 250 000 steps and run length of 1 000 000.
151 Three independent runs were done to verify the convergence of results. The number of groups
152 (*K*) analysed ranged from 1 to 10, with 10 independent runs per *K*. The best K value was
153 identified by maximum value of ΔK (Evanno *et al*., 2005) as implemented in Structure
154 Harvester (Earl and von Holdt, 2012). The Pophelper (Francis, 2016; http://pophelper.com/)
155 was used to summarize the results of optimal *K* value and generate the bar plot figures.
156
157 ***Mating system and Gene flow***
158 To analyse whether there is gene flow among populations, we used the alleles of adult
159 individuals (n = 46), mother plants (n = 11) and their progenies (n = 125). Before performing
160 paternity analyses, we visually inspected for mismatches between the parent plants and their
161 offsprings. All genotyped individuals were included because we did not find exclusion of any
162 mother.
163 The crossing rate was evaluated through MLTR 3.4 (Multilocus Mating System)
164 program (Ritland, 2002, 2004). All nine loci were analysed based on the models mixed
165 mating (Ritland and Jain, 1981) and correlated crosses (Ritland, 1989). The estimated
166 parameters were: inbreeding coefficient of maternal parents (*F*); multilocus auto crossing rate
167 (t_m); single locus auto crossing rate (t_s); the difference between them $t_m - t_s$; correlation of
168 paternity multilocus (r_{pm}); correlation of paternity single locus (r_{ps}); the difference between
169 them $r_{pm} - r_{ps}$; and correlation of selfing (r_s). To estimate the standard error of the parameters,
170 we used bootstrap with 1000 resembling and 95% confidence interval in MLTR.
171 We also estimated the inbreeding depression (δ) to *P. secreta* offspring using the
172 method of Goodwillie *et al.* (2005), as:

$$\delta = 1 - 2[(1\text{-}s)F / s(1 - F)],$$

where the s $(1 - tm)$ and F parameters were obtained from MLTR results (see Table 2). In Cervus 3.0.6 software (Kalinowski *et al.*, 2007; http://helios.bto.ed.ac.uk/evolgen), using the maximum likelihood based method, we run the paternity assignment test approach (Marshall *et al.*, 1998). We identified the most likely pollen donor for each offspring and determined the mating structure and pollen dispersal distance. The most likely pollen donor was determined by the Δ statistical. The significance of Δ (critical Δ) was obtained by 10 000 rounds of simulation using the allele frequencies of loci of the adults. The simulation assessed the level of confidence through the actual parentage analysis. After, we carried out the parentage analysis to assign the candidate father of each offspring. Individual with the highest Δ value was accepted as father to a seed if the difference between its LOD score (logarithm of likelihood ratios) and the second most likely candidate's LOD score was above the critical Δ (threshold value). We used the following parameters in this analysis: 10 000 repetitions; 0.9324 proportion of loci typed; 95% (strict) and 85% (relaxed) levels of confidence. We considered 90% of parents in the area as sampled and 1% of genotyping error. The minimum of loci necessary to determine the paternity of a seedling was fixed at six.

RESULTS

Characterization of microsatellite loci

All pairs of loci were in linkage equilibrium (almost all $P < 0.001$, Bonferroni's adjusted value for a nominal level of 5 %), and most of the 12 microsatellite loci displayed high levels of polymorphism and diversity. We detected 68 alleles among the adult individuals (Table 3). The observed heterozygosity (H_O) was lower than the expected (H_E), showing a deficit of heterozygotes in relation to the Hardy–Weinberg equilibrium ($P < 0.005$). Only for PM183 locus, H_O was larger than H_E. For almost all loci, the inbreeding coefficient (F_{IS}) was positive

198 and significant (Table 3). The high combined paternity exclusion probability (*QC* = 0.9963)
199 and the low combined probability of identity ($IC = 2.407 \times 10^{-11}$) showed that this set of
200 microsatellite loci is suitable for parentage analyses in *P. secreta* (Table 3). Generally, we
201 obtained the same results analysing the seven grouped populations (see Supplementary Data
202 Table S1). The P1_GP_Mirante and P7_PS_P219 populations presented higher diversity
203 indices by number of alleles. All populations except P5_PS_PSM showed private alleles
204 (Table S1).
205
206 ***Population structure***
207 In AMOVA analysis we observed high genetic variation among the seven populations (~25%;
208 P< 0.05), whereas the genetic variation within population was ~75%. When we considered
209 three hierarchical levels (two groups: Pedra do Segredo and Galpão de Pedra populations),
210 similar patterns of differentiation among populations and within population were observed
211 (~22% and 74%, respectively; P<0.05) and no significant genetic differentiation was observed
212 between two groups (considering Pedra do Segredo and Galpão de Pedra groups; $F_{CT} = 0.03$;
213 P>0.05). The measurement of genetic differentiation among the seven populations through
214 pairwise estimators of F_{ST} showed significant and medium to high genetic differentiation only
215 in nine comparisons (Table 4). To these nine comparisons, the F_{ST} values ranged from 0.176
216 (PS_P219 x GP_Mirante) to 0.458 (GP_P214 x GP_221). Note that the highest F_{ST} value was
217 obtained between the geographically closest populations (Fig. 1).
218 The results of population structure based on coalescent model implemented in
219 Structure software are presented in Figure 2. In this analysis, we observed that the most likely
220 number of clusters was *K = 4*. No geographical structure was observed in the sampled area of
221 *P. secreta,* with all populations presenting the four genetic components. Moreover, several
222 individuals presented mixed ancestry. On the other hand, some individuals were

223 homogeneous, especially individuals from populations 4 and 7 (Fig. 2).

224 ***Breeding structure and pollen dispersal***

225 The mating parameters estimated by the MLTR program are summarized in Table 2. The
226 estimates of multilocus auto crossing rate (t_m) were significantly lower than the unit (1.0),
227 indicating that *P. secreta* has a mixed crossing system with predominance of self-fertilization.
228 The single locus auto crossing rate (t_s) was significantly lower than the multilocus auto
229 crossing rate. The difference between the rates ($t_m - t_s$) was equally significant, suggesting that
230 biparental inbreeding (endogamy resulting from mating between relatives) contributed greatly
231 to the auto crossing rate. The inbreeding coefficient of maternal parents (F) indicated that
232 there was an excess of homozygotes and high inbreeding in the parental population. Another
233 measure that indicates the occurrence of crosses between relatives is the difference between
234 the correlation of paternity single-locus and the correlation of paternity multilocus ($r_{ps} - r_{pm}$).
235 This difference was significantly lower than zero, indicating that in addition to correlated
236 crosses, some of the pollen donor plants were related one to each other. The correlation of
237 selfing (r_s) was significantly greater than zero, as well as the estimate of the correlation of
238 paternity multilocus (r_{pm}), indicating that occurred crosses between unrelated individuals and
239 that part of the progenies were full-siblings. The estimate of correlation of paternity single-
240 locus (r_{ps}) was also significantly lower than zero. The paternity correlation suggests that most
241 progenies are full-sibs. Inbreeding depression value was high suggesting that fitness of *P.*
242 *secreta* progeny can be affected by inbreeding depression (Table 3).
243 In the paternity test of 125 offspring from 11 mother plants from the sampled area, we
244 obtained 83% of paternity assignments considering strict confidence (95%, critical $\Delta = 1.24$),
245 and 100% considering relaxed confidence (85%, critical $\Delta = 0.00$), father given known
246 mother. About 14% of adult individuals contributed to the pollination process in the analysed
247 generation. Paternity analysis also showed that the pollination process was predominantly

248 selfing and all observed outcrossing occurred within the same population that the mother
249 plant. Thus, the pollen dispersal always occurred in zero distance (Table 5) showing local
250 pollination process. Interestingly, we can see that in population four have two distinct genetic
251 components, yellow and orange (Fig. 2). The individual 790 (Fig. 2 highlighted with *) has
252 the two genetic components. This shows that although pollination is restricted to one site, new
253 combinations of genetic components may occur.
254
255 **DISCUSSION**
256 In this study, we investigated the mating system of the rare and endemic *P. secreta* in its wide
257 distribution area. It is currently ranked in the official listings as threatened with extinction
258 because of its restricted distribution (Turchetto *et al*., 2016). Few populations and
259 reproductive adults were found (7 and 46, respectively; see Fig. 1 and Table 1) and, despite
260 the number of individuals changes seasonally, the sites are almost constantly observed. In
261 addition, these populations occur in rocky outcrops associated with rock vegetation
262 (vegetation composed mainly of Bryophyta and species of the families Bromeliaceae and
263 Cactaceae) that are isolated one to each other by hillside forest. *Petunia secreta* is pollinated
264 by a small bee (Rodrigues et al., unpublished data) and, as in all *Petunia* species (Stehmann
265 *et al*. 2009), the seeds are dispersal by autochory, which is short distance of cross-pollination
266 and dispersion of seeds (Table 5).
267 Our results showed that there is no gene flow among populations. The paternity
268 analysis revealed 88% of self-pollination in the progenies (Table 5), and just few pollination
269 events are suggested by high paternity correlation (Table 3). The allele frequencies evaluation
270 shows a low heterogeneity index (Table 3). The *P. secreta* pollinator is a small bee,
271 *Pseudagapostemon* sp. Schrottky [Stehmann *et al.*, 2005; Rodrigues *et al.* (unpublished
272 data)]. According to literature the maximum foraging distances of small bees is 100-200 m

(Gathmann and Tscharntke, 2002; Zurbuchen *et al.*, 2010). The average distance between the populations was ~ 200 m (Fig. 1) that corresponds to the maximum flight distance mentioned in the literature. Rodrigues *et al.* (unpublished data) observed that *Pseudagapostemon* sp. arrives in the flower without loading of pollen and collects all the pollen at one time during a visit (in 100% of visits observed), which favour self-pollination. They also observed that *P. secreta* does not do spontaneous pollination, therefore it is dependent of the pollinator. This shows that thus, although the distance between populations is reasonable to pollinator flies across them, the bees' behaviour indicates that there is a return to the nest at each collection and consequently a decrease in cross-pollination between individuals from different populations. The just collected-pollen position favour the contact between pollen and stigma from the same flower, promoting the self-pollination. Controlled experiments of pollination conducted at greenhouse showed that *P. secreta* is dependent from a pollinator because no fruit was produced by flowers in spontaneous selfing (Rodrigues *et al.,* unpublished data) according with some herkogamy levels observed in *P. secreta*.

The autochory in *P. secreta* also negatively influences the gene flow among populations, because it improves the probability of only related individuals grow together in the same area. The difference between the rates ($t_m - t_s$) and the correlations ($r_{ps} - r_{pm}$) suggests the occurrence of crosses between individuals related within the populations (inbreeding). Biparental inbreeding, in turn, can trigger endogamic depression. Our results showed that inbreeding depression could affect the fitness of progeny ($\delta = 0.856$).

Although its rarity and high levels of self-pollination and inbreeding, *P. secreta* presents high genetic diversity, mainly when compared to its congeneric endemic species *P. exserta* (Turchetto *et al.*, 2016). We believe that the seed bank due to seed dormancy can be a plausible hypothesis for maintaining the diversity of *P. secreta*. This species is annual (Stehmann and Semir, 2005) and Rodrigues *et al.* (unpublished data) observed that, year to

298 year, the individuals are not observed in the same place but nearby and, in a second year
299 individuals again appear in the first place (personal observations). This could suggest the
300 formation of a seed bank that sustains a temporal gene flow, causing individuals with different
301 genotypes to have an opportunity, albeit small, for crossing. This is according to observed in
302 genetic structure within populations (Fig. 2). We observed in POP 4 (Fig. 2) that there was
303 cross-pollination between individuals from different genetic clusters but from the same
304 population. In fact, dormancy is a key trait for survival and fitness (Linkies *et al.*, 2010)
305 influencing natural population dynamics (Bewley *et al*., 2013; Smýkal *et al*., 2014).
306 Frequently, the seeds from the same plant present different degree of dormancy, which is
307 reflected in appearance of seeds as colour, size or thickness of coat (Bewley *et al*., 2013).
308 Although *P. secreta* has high genetic variability (Turchetto *et al*., 2016), the
309 heterozygosity values of the populations were very low (Table S1) and inbreeding depression
310 would affect the fitness of this species in the future. The decrease in heterozygosity and
311 increased inbreeding may result in the accumulation of deleterious alleles, leading to a
312 decrease in fertility rates, increased mortality, and reduced plant growth rate (Young *et al.*,
313 1996). The reduction of heterozygosity leads to a decrease in the adaptive value (Young *et al.*,
314 1996). Even more so because the *P. secreta* populations are isolated (naturally fragmented), it
315 is expected that this species should suffer with a low proportion of polymorphic loci and a
316 reduction in the number of alleles per locus in these populations. It can also reduce long-term
317 responses of species to changes imposed by selection pressures, which may increase the
318 probability of species extinction as suggested for other species in similar conditions (Young *et*
319 *al.*, 1996; O'Grady *et al.*, 2006). The fire practices to clean the environment used in the region
320 may be a causal factor putting the species at risk of extinction. This study could come useful
321 to establish a management plan to maintain the genetic diversity and the species per se. So,
322 because maintaining genetic diversity should be the main objective of conservation actions

(Frankham, 2010), we suggests to the local authorities mainly to promote educational training in the region as a way to control fire and economic activities that may lead to the species disappearance collaborating, thus, to preserve the natural habitat of this species. Another strategies is collecting seeds from as many individuals as possible and stored in seed banks and living collections, thus promoting regular genetic monitoring to restore eventual stochastic losses.

CONCLUSIONS

In view of this scenario, we can affirm that *P. secreta* is an species that reproduces essentially through self-pollination mediated by a pollinator. Decreased heterozygosity and increased inbreeding may result in decreased adaptability of this species to withstand selection pressures in the region. This species needs to receive attention from local authorities to ensure awareness of its preservation. *P. secreta* is a species of great interest for evolutionary studies since it forms a clade with *P. exserta* and *P. axillaris*, which is suggested having a history of speciation mediated by pollinator differentiation (Fregonezi *et al.*, 2013).

SUPPLEMENTARY DATA

Supplementary data are available online at www.aob.oxfordjournals.org and consist of the following: Table S1: Characterization of the nine microsatellites per locus and per collection site of *Petunia secreta*.

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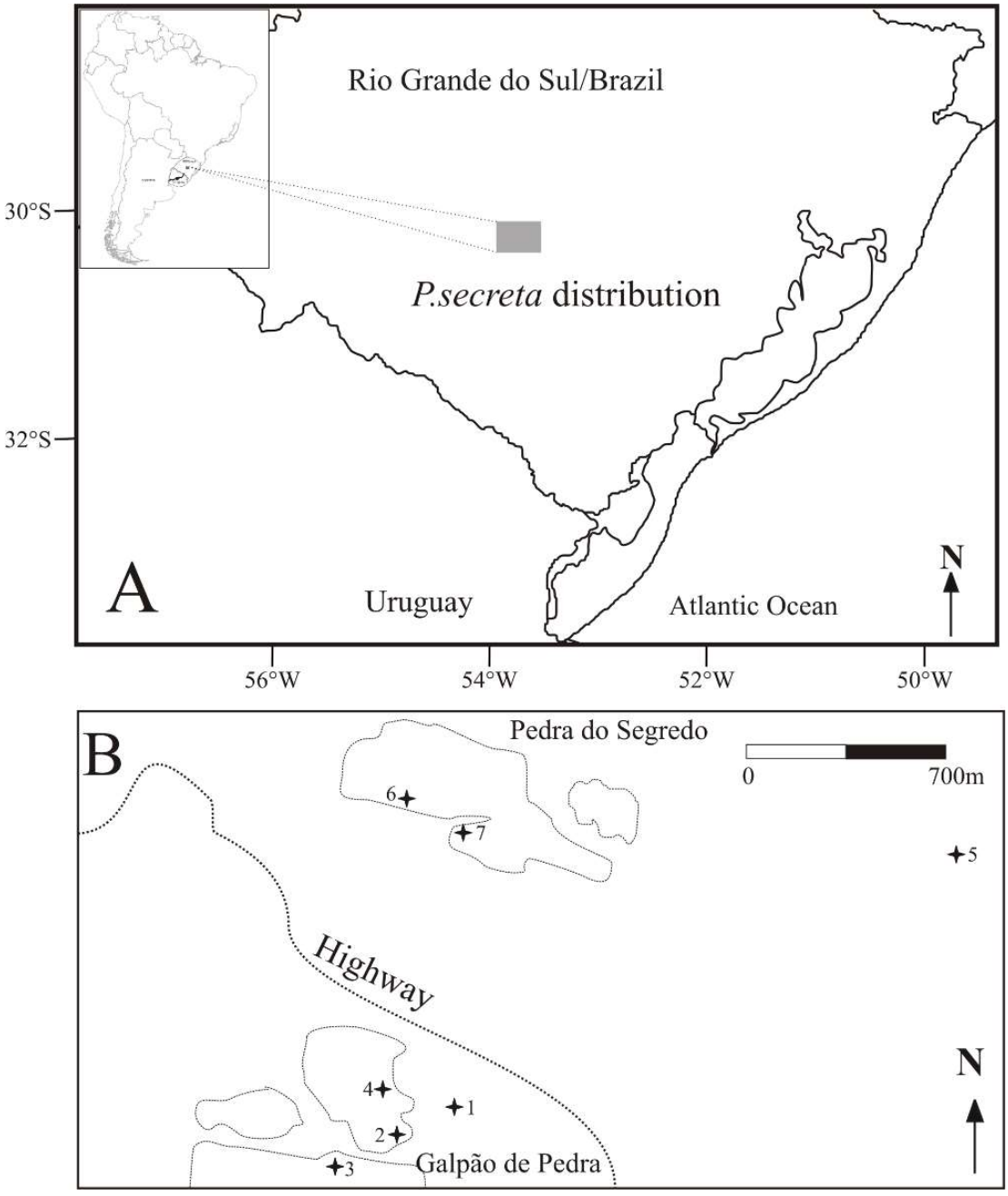

Figure 1. *Petunia secreta* collection sites. Geographical coordinates are listed in Table 1.
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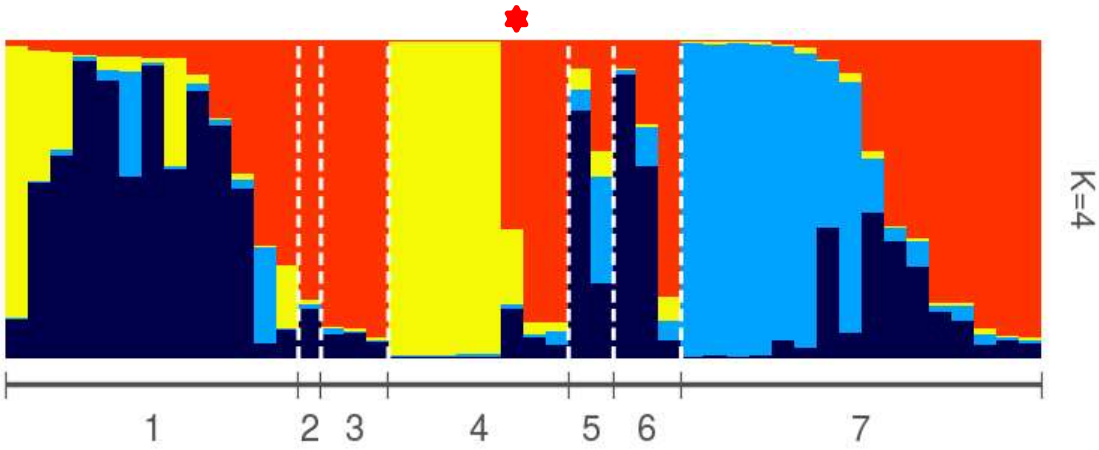

Figure 2. STRUCTURE bar plot under an admixture coefficients model based on nine microsatellite loci and seven populations of *Petunia secreta*. Bars represent individuals, and black vertical lines represent each population; different colors indicate $K = 4$ genetic components representing individual membership of one of the genetic clusters.

Table 1. Information on *Petunia secreta* analysed populations.

Population	ID population	*n*	Geographic coordinate
Pop 1	**GP_Mirante (4)**	13	30° 32' 45.92" S 53° 33' 00.97" W
Pop 2	GP_Corda	1	30° 32' 48.67" S 53° 33' 07.39" W
Pop 3	GP_P214	3	30° 32' 52.00" S 53° 33' 16.30" W
Pop 4	**GP_P221 (5)**	8	30° 32' 43.60" S 53° 33' 10.40" W
Pop 5	PS_PSM	2	30° 32' 18.70" S 53° 31' 52.80" W
Pop 6	**PS_Estalac (1)**	3	30° 32' 09.64" S 53° 33' 07.19"W
Pop 7	**PS_P219 (1)**	16	30° 32' 13.00" S 53° 32' 59.00"W
Overall	7 **(11)**	46	3.000m^2

The populations where the mother plants were chosen are in bold, with the number of mother plants collected by population in parentheses.

Table 2. Mating system parameters of *P. secreta* estimated from 10 families by MLTR analysis.

Parameters	**Estimated (s.d.)**
Parental F	0.409 (0.099)
Crossing multilocus (t_m)	0.094 (0.033)
Crossing single locus (t_s)	0.030 (0.013)
Difference ($t_m - t_s$)	0.064 (0.023)
Correlation of paternity singlelocus (r_{ps})	-0.999 (0.173)
Correlation of paternity multilocus (r_{pm})	0.973 (0.455)
Difference ($r_{ps} - r_{pm}$)	-1.972 (0.539)
Correlation of selfing among families (r_s)	0.814 (0.372)
Inbreeding depression (δ)	0.856

509 **Table 3.** Characterization of nine microsatellite loci of *Petunia secreta* based on 46 adult

510 individuals sampled in the Pedra do Segredo, Serra do Sudeste, Rio Grande do Sul/Brazil.

Locus	A	*AR*	H_E	H_O	F_{IS}	*Q*	*I*
PM184	4	3.948	0.462	0.152*	0.673*	0.179	0.416
PM88	7	6.902	0.640	0.196*	0.697*	0.424	0.160
PM177	18	17.285	0.916	0.351*	0.620*	0.753	0.027
PM167	8	7.579	0.792	0.432*	0.458*	0.518	0.106
PM183	8	7.662	0.778	0.861*	-0.107	0.571	0.083
PM188	8	7.603	0.794	0.326*	0.592*	0.456	0.140
PM173	5	4.643	0.567	0.196*	0.658*	0.302	0.253
PM195	5	3.902	0.520	0.239*	0.543*	0.195	0.379
PM192	5	5.000	0.758	0.188*	0.756*	0.504	0.113
Overall	68		0.572	0.330*	0.531*	$QC = 0.9963$	$IC = 2.407 \times 10^{-8}$

511 A, number of alleles; AR, allele richness; HE, expected heterozygosity; HO, observed
512 heterozygosity (all values were significant, P<0.005, Bonferroni's adjusted P-value for a
513 nominal level of 5 %); F_{IS}, endogamy coefficient; Q, probability of paternity exclusion; QC,
514 combined probability of paternity exclusion. I, probability of genetic identity; IC, combined
515 probability of genetic identity.
516

517 **Table 4:** Population differentiation of *Petunia secreta* measured through F_{ST}.

	GP_Mirante	GP_Corda	GP_P214	GP_P221	PS_PSM	PS_Estalac	PS_P219
GP_Mirante	0.000						
GP_Corda	0.324	0.000					
GP_P214	0.333*	0.606	0.000				
GP_P221	0.226*	0.575	0.458*	0.000			
PS_PSM	0.192	0.513	0.568	0.365*	0.000		
PS_Estalac	0.021	0.280	0.326	0.341*	0.165	0.000	
PS_P219	0.176*	0.320	0.373*	0.281*	0.106	0.183*	0.000

518 *P<0.05

519

520 **Table 5:** Results of paternity analysis by offspring of *Petunia secreta* in sampled area.

Population ID	Mother Plant ID	*n*	Assignment ≥85% confidence	Selfing	Pollen Flow	N_e	Max. Distance pollen
GP_Mirante	MP 53	15	15	13	2	2	0*
GP_Mirante	MP 55	8	8	8	0	1	0
GP_Mirante	MP 56	15	15	15	0	1	0
GP_Mirante	MP 62	15	15	14	1	2	0*
GP_P221	MP 786	14	14	14	0	1	0
GP_P221	MP 787	15	15	7	8	3	0*
GP_P221	MP 788	6	6	3	3	2	0*

GP_P221	MP 789	12	12	11	1	2	0*
GP_P221	MP 790	4	4	4	0	1	0
PS_Estalac	MP 59	15	15	15	0	1	0
PS_P219	MP 73	6	6	6	0	1	0
Total	11	125	125 (100%)	110 (88%)	15 (12%)	17 (13.6 %)	0

*outcrossing occurs between individuals from the same population that mother plant. *n* is the number of seedlings per mother plant; N_e number of pollen donors. Values of selfing, pollen flow, and N_e are based on percentage of assignments with ≥ 85% confidence.

Table S1: Characterization of the nine microsatellites per locus and per collection site of *P. secreta*.

Site/loci		PM184	PM88	PM177	PM167	PM183	PM188	PM173	PM195	PM192	Average
GP_Mirante											
	N	2	5	9	5	5	6	3	2	4	4.56
	E	0	0	1	0	0	0	1	0	0	0.22
	H_E	0.323	0.508	0.815	0.758	0.743	0.689	0.557	0.471	0.583	0.605
	H_O	0.077	0.308	0.417*	0.545	0.917	0.308*	0.308	0.231	0.250	0.373
	F_{IS}	0.769	0.404	0.500	0.290	-0.247	0.564	0.458	0.520	0.582	0.426
GP_Corda											
	N	1	1	NA	2	2	1	1	1	2	1.22
	E	0	1	0	0	0	0	0	1	0	0.22
	H_E	NA	NA	NA	1.000	NA	NA	NA	NA	1.000	0.222
	H_O	NA	NA	NA	1.000	NA	NA	NA	NA	1.000	0.222
	F_{IS}	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
GP_214											
	N	2	2	5	1	2	2	2	2	1	2.11
	E	0	0	2	0	1	0	1	1	0	0.56
	H_E	0.333	0.333	0.933	NA	0.600	0.533	0.600	0.533	NA	0.429
	H_O	0.333	0.333	0.667	NA	1.000	0.667	0.333	0.667	NA	0.444
	F_{IS}	0.000	0.000	0.333	NA	-1.000	-0.333	0.500	-0.333	NA	- 0.833
GP_P221											
	N	2	3	4	3	4	4	1	2	4	3.00
	E	0	0	2	1	0	0	0	0	0	0.33
	H_E	0.233	0.492	0.561	0.492	0.692	0.708	NA	0.458	0.758	0.488
	H_O	0.000	0.125	0.500	0.500	0.714	0.500	NA	0.375	0.143*	0.317
	F_{IS}	1.000	0.759	0.118	-0.018	-0.034	0.309	NA	0.192	0.000	0.258
PS_PSM											
	N	1	2	2	2	3	2	1	1	2	1.78
	E	0	0	0	0	0	0	0	0	0	0.00

	H_E	NA	0.667	0.667	0.667	0.833	0.667	NA	NA	0.500	0.445
	H_O	NA	0.000	0.000	0.000	1.000	0.000	NA	NA	0.500	0.167
	F_{IS}	NA	1.000	1.000	1.000	-0.333	1.000	NA	NA	0.000	0.407
PS_Estalac											
	N	2	3	4	2	4	4	2	2	2	2.78
	E	0	0	1	0	1	0	0	0	0	0.22
	H_E	0.333	0.800	0.867	0.600	0.867	0.800	0.333	0.533	0.533	0.630
	H_O	0.333	0.000	0.333	0.333	0.667	0.667	0.333	0.667	0.000	0.370
	F_{IS}	0.000	1.000	0.667	0.500	0.273	0.200	0.000	-0.333	1.000	0.367
P7_PS_P219											
	N	4	5	9	5	4	5	3	2	2	4.33
	E	1	1	1	2	0	1	1	0	0	0.78
	H_E	0.609	0.700	0.909	0.732	0.637	0.690	0.462	0.062	0.356	0.573
	H_O	0.250*	0.187*	0.182*	0.437	0.867	0.187*	0.187	0.062	0.000	0.173
	F_{IS}	0.597	0.738	0.808	0.410	-0.379	0.735	0.602	0.000	1.000	0.501
TOTAL											
	N	4	7	18	8	8	8	5	4	5	7.44
	E	1	2	7	3	2	1	3	2	0	2.33
	H_E	1.831	3.500	4.752	4.249	4.372	4.087	1.952	2.057	3.730	3.392
	H_O	0.993	0.953	2.099	2.815	5.165	2.329	1.161	2.002	1.893	2.157
	F_{IS}	2.366	3.901	3.426	2.182	- 1.72	2.475	1.56	0.046	1.582	1.579

N, number of alleles; E, number of private alleles; R, allele richness; H_E, expected heterozygosity; H_O, observed heterozygosity; F_{IS}, inbreeding coefficient. * Hardy–Weinberg equilibrium deviation significance per locus per taxon after Bonferroni correction at $P = 0.05$.

CAPÍTULO VI
DISCUSSÃO GERAL

Com o intuito de agregar mais conhecimento aos estudos evolutivos no gênero *Petunia*, este trabalho avaliou a história reprodutiva da espécie rara *Petunia secreta* através de diferentes abordagens. Os aspectos investigados referentes ao seu sistema reprodutivo e sua dinâmica genética serão relevantes tanto para o entendimento dos caminhos evolutivos dentro do clado tubo longo quanto para a conservação de *P. secreta*. Os resultados deste trabalho podem contribuir para o entendimento da manutenção do sucesso de espécies raras na natureza.

Através de horas de observação (176 horas; Capítulo II), nossos resultados comprovaram que a espécie de abelha coletora de pólen *Pseudagapostemon* sp. é o polinizador efetivo de *P. secreta* como sugerido por Stehmann & Semir (2005). O que este trabalho acrescentou de conhecimento a essa informação foi demonstrar como *Pseudagapostemon* sp. é de extrema importância para a reprodução de *P. secreta* e como esse polinizador influencia o sistema reprodutivo. Primeiramente, os testes de sistema reprodutivo, especialmente o ensacamento de flores intactas, mostraram que *P. secreta* não possui polinização espontânea (Capítulo IV), o que confirma sua dependência do polinizador para a fecundação. Outro aspecto importante é o comportamento do polinizador durante sua visita à flor, pois é a peça chave para entender o papel desse polinizador sobre o sucesso reprodutivo da planta (Rathcke, 1992). A arquitetura das estruturas reprodutivas (com o estigma posicionado acima das anteras) e o comportamento de coleta de *Pseudagapostemon* sp. durante a visita (se apoiando sobre as anteras e o estigma enquanto realizava a coleta de todo o pólen disponível (Figura 1; Capítulo II), comprovam a eficiência desta espécie de abelha para a polinização de *P. secreta*. As observações também revelaram que as abelhas chegavam à flor sem carregamento polínico e efetuavam a coleta de todo o pólen de uma só vez, sugerindo fortemente que o pólen é descarregado no ninho a cada visita floral (Capítulo II). Esse comportamento do polinizador reduz a possibilidade de polinização cruzada, como

podemos comprovar pelos resultados de análise de paternidade que mostraram que 88% das progênies foram resultantes de autopolinização (Capítulo V). Com isso, apesar de *P. secreta* possuir uma disposição das estruturas reprodutivas que favorece a polinização cruzada, o comportamento do polinizador direciona o sistema reprodutivo para essencialmente autopolinização. Embora *Pseudagapostemon* sp. tenha condições de transitar entre as populações de *P. secreta,* que se localizam a uma distância média de cerca de 200 metros (Figura 1; Capítulo V), mesma faixa atribuída na literatura para distância máxima de forrageio de abelhas pequenas (Gathmann & Tscharntke 2002; Zurbuchen *et al.*, 2010), o comportamento observado de descarregamento de pólen a cada visita floral faz com que o fluxo gênico entre as populações também seja dificultado, comprovado pelos resultados de análise de paternidade que mostraram não haver fluxo gênico entre as populações.

Assim como todas as espécies do clado tubo curto de *Petunia* que são polinizadas por abelhas, a corola de *P. secreta* também reflete raios ultravioleta (Figura 2B capítulo 2). Embora os estudos moleculares para desvendar o cenário evolutivo que levou *P. secreta* a refletir UV ainda estejam em andamento, a condição de refletância certamente favoreceu a polinização por abelhas em *P. secreta*. A diferença de cor do pólen (azul nas espécies de tubo curto e amarelo em *P. secreta*) e, provavelmente, os aromas podem estar envolvidos na atração de diferentes gêneros de abelhas que polinizam essas espécies (Capítulo III). A interação inseto-planta nas espécies de tubo curto é bem mais complexa do que em *P. secreta*. As abelhas utilizam as flores das espécies de tubo curto como local de acasalamento, abrigo, fonte de néctar e as fêmeas ainda coletam o pólen (Wittmann *et al.,* 1990). Em *P. secreta*, *Pseudagapostemon* sp. apenas coleta pólen, pois o tubo floral é muito estreito e não há espaço suficiente para que elas adentrem até os nectários (Figura 2D, Capítulo 2). Também não observamos *Pseudagapostemon* sp. utilizando as flores como repouso ou local de acasalamento, embora outras espécies de abelhas o façam em *P. secreta* sem que estejam

envolvidas em sua polinização (Tabela 1, Capítulo 2).

Os experimentos de polinização conduzidos em casa de vegetação revelaram que *P. secreta* não produz frutos por apomixia, não se autofecunda espontaneamente e é autocompatível (Capítulo IV). Diferentemente de *P. exserta* que possui uma hercogamia temporária, pois as anteras ultrapassam o estigma durante o desenvolvimento floral e acabam por promover auto polinização (dados não publicados), *P. axillaris* e *P. secreta* mantém sua hercogamia ao longo de todo o desenvolvimento floral, mecanismo esse que impede a autopolinização espontânea e favorece a polinização cruzada. Todavia, há uma grande diferença entre o sistema reprodutivo das espécies de tubo curto polinizadas por abelhas e *P. secreta*. Nas primeiras, além de serem autoincompatíveis, as abelhas visitam várias flores em um mesmo evento de forrageio (dados não publicados) e, além do pólen, adentram as flores para coleta de néctar (Wittmann *et al.* 1990), promovendo assim a polinização cruzada.

As sementes de *P. secreta* são dispersas por autocoria e todas as sementes dos testes de polinização manual germinaram igualmente (Capítulo IV), indicando não haver diferenças quanto à viabilidade relacionada ao tipo de fecundação. A autocoria promove a ocorrência de indivíduos aparentados e mantem os genótipos limitados dentro de uma mesma área. Isso é refletido nos resultados de análise de paternidade que mostram que os cruzamentos existentes foram por endogamia (Capítulo V). Por outro lado, a dinâmica de surgimento de indivíduos nos locais ao longo dos anos observados sugere que há um banco de sementes que pode estar mantendo a espécie. Esse banco pode estar proporcionando a aleatoriedade genotípica necessária para manter a variabilidade ao longo do tempo, como observado por Turchetto *et al.* (2016).

O experimento conduzido com análise de progênies mostrou que, apesar de *P. secreta* ter um sistema misto de reprodução, a autopolinização foi predominante e também que os cruzamentos endogâmicos são frequentes. O aumento da autopolinização e da endogamia

pode resultar no acúmulo de alelos deletérios ao longo do tempo e até provocar uma diminuição nas taxas de fecundidade (Young *et al*., 1996). Devido à fragmentação da distribuição das populações e à ausência de fluxo gênico entre elas (Capítulo V), o isolamento por diversas gerações também pode causar a perda de alelos em decorrência da deriva genética. Além disso, o baixo índice de heterozigosidade em *P. secreta* (Capítulo V) pode levar à diminuição do valor adaptativo (Young *et al*., 1996) e reduzir as respostas às mudanças impostas pelas pressões de seleção, aumentando com isso a probabilidade de extinção da espécie (O'Grady *et al*., 2006). Considerando que o único local preservado na região é o Parque Municipal da Pedra do Segredo e este é muito pequeno, onde apenas poucas populações de *P. secreta* foram encontradas, podemos dizer que a condição de ameaça de *P. secreta* é evidente não somente por sua distribuição restrita, mas também pelo baixo nível de heterozigosidade encontrada. Por isso é urgente que a existência de *P. secreta* seja amplamente divulgada à comunidade do entorno da Pedra do Segredo, promovendo assim a valorização e conscientização da espécie como patrimônio natural de Caçapava do Sul e divulgação da importância de preservar o ambiente onde esta ocorre fora da proteção do parque.

CAPÍTULO VII
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