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PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS BIOLÓGICAS
(BIOLOGIA VEGETAL)

DIFERENCIAÇÃO INTRAESPECÍFICA NA REPRODUÇÃO E INTERAÇÕES PLANTA-POLINIZADOR EM POPULAÇÕES NATURAIS DE *TREMBLEYA LANIFLORA* (MELASTOMATACEAE)

Natalia Costa Soares

Tese apresentada ao Instituto de Biociências do Câmpus de Rio Claro, Universidade Estadual Paulista, como parte dos requisitos para obtenção do título de doutor em Ciências Biológicas (Biologia Vegetal).

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TÍTULO DA TESE: *Diferenciação intraespecífica na reprodução e interações planta-polinizador em populações naturais de trembleya laniflora (melastomataceae)*

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Rio Claro, 13 de novembro de 2017

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“A vida é arte do encontro, embora haja tanto desencontro pela vida.” (Vinícius de Morais/ Baden Powell)

RESUMO

Indivíduos coespecíficos utilizam de forma diferente os recursos do nicho disponíveis às suas populações, e essas variações intraespecíficas (inter e intrapopulacionais) influenciam muitos processos ecológicos, principalmente as interações planta-animal. Em plantas com polinização biótica, a floração (época e intensidade) é uma importante adaptação aos períodos de maior abundância dos vetores de pólen, e está relacionada à atratividade aos polinizadores. A relação evolutiva entre polinizadores e caracteres florais das espécies e, ocorrência das "Síndromes de polinização" vem sendo historicamente testada, e geralmente, é confirmada a existência de uma relação direta entre um conjunto de caracteres florais e os polinizadores principais das espécies. Neste contexto, utilizando como modelo de estudo *Trembleya laniflora* Cong., uma espécie de Melastomataceae endêmica dos campos rupestres da porção sul da Cadeia do Espinhaço, Minas Gerais (Brasil), com populações naturalmente isoladas em afloramentos rochosos, e que apresenta características florais (flores grandes e brancas) que diferem do padrão observado para a família, tivemos como objetivo deste trabalho: (i) entender a importância ecológica e evolutiva dos distintos caracteres florais de *Trembleya laniflora* para a sua polinização e sucesso reprodutivo; (ii) avaliar a ocorrência de variação intraespecífica interpopulacional na ecologia reprodutiva da espécie, testando os fatores bióticos e abióticos que determinam tais diferenças e sua relevância para o sucesso reprodutivo populacional; e (iii) avaliar a variação intraespecífica interpopulacional na fenologia de floração e nas interações planta-polinizador, acessando como a especialização no nível individual e populacional afetam o sucesso reprodutivo. Para isso, observamos a fenologia reprodutiva (floração e frutificação), biologia floral, visitantes florais e polinizadores, e testamos o sistema reprodutivo da espécie; observamos e testamos as variações da fenologia reprodutiva, biologia floral e ecologia da polinização entre três populações localizadas em áreas de distintas altitudes da Serra do Cipó, MG (Cedro, 1101m; Pedra do Elefante, 1255m; Quadrante 16, 1303m), avaliando quais fatores bióticos e/ou abióticos melhor explicam o sucesso reprodutivo populacional; e avaliamos os efeitos da generalização/especialização no uso dos recursos dos nichos fenológico (tempo) e de polinização (interações indivíduo de planta-espécie de polinizador) para o sucesso reprodutivo individual e populacional. Características florais e de biologia reprodutiva (antese noturna) de *T. laniflora* foram associadas a um sistema de polinização especializado mediado por abelhas grandes, vibradoras, com comportamento de forrageio crepuscular. Populações espacialmente isoladas e sob distintas condições microclimáticas diferiram em sua biologia reprodutiva (inícios e picos fenológicos, hora da antese, longevidade floral, e frequência de visitas legítimas) e sucesso reprodutivo. Fatores abióticos, principalmente a temperatura, determinaram as diferenças nas datas fenológicas entre as populações, enquanto que fatores bióticos, relacionados às interações planta-polinizador, influenciaram na produção de frutos maduros e no sucesso reprodutivo. Finalmente, populações compostas por indivíduos mais generalistas em suas interações com polinizadores e sincrônicos em sua atividade de floração (época, duração e intensidade) apresentaram maior sucesso reprodutivo. No nível individual, plantas mais centrais e generalistas em suas interações com os polinizadores também apresentaram maior sucesso. Portanto, a generalização e baixo grau de especialização no uso dos recursos, tempo de floração e polinizadores, pelos indivíduos de *Trembleya* exerce um

papel fundamental para a conservação das interações planta-polinizador e, positivamente, afetam o sucesso reprodutivo desta espécie altamente especializada para seu sistema de polinização.

ABSTRACT

Conspecific individuals vary in their use of available resources within the population, and intraspecific variations (inter and intrapopulation) in species traits play a fundamental role in many ecological processes, primarily the plant-animal interactions. The flowering activity (time and intensity) is an important adaptation to the periods of larger abundance of pollen vectors, enhancing the plant attractiveness to pollinators. The evolutionary relationship between pollinators and floral traits and the occurrence of "Pollination Syndromes" has been historically tested, and the existence of a direct relationship between a set of floral traits and the main pollinators has been generally confirmed. In this context, using as study model *Trembleya laniflora* Cong. (Melastomataceae), an endemic species, naturally isolated on rock outcrops from the rupestrian grasslands of South Espinhaço Range, Minas Gerais (Brazil), presenting floral characteristics (large and white flowers) that differ from the pattern observed for the family, we aimed: (i) to investigate the ecological and evolutionary importance of the distinct floral traits of *Trembleya laniflora* for its pollination and reproductive success; (ii) to observe and evaluate the occurrence of interpopulational intraspecific variation in the reproductive ecology of the species, testing the biotic and abiotic drivers for their reproductive success; and (iii) to evaluate intrapopulational intraspecific variation in flowering phenology and plant-pollinator interactions, accessing as specialization at individual and population levels are related to reproductive success. For this, (i) we observed the reproductive phenology (flowering and fruiting), floral biology, flower visitors and pollination, and test the reproductive system of the species; (ii) we tested for differences in the reproductive phenology, floral biology and pollination ecology among three populations located at different altitudes of *Serra do Cipó*, MG (Cedro, 1101m; Pedra do Elefante, 1255m; Quadrante 16, 1303m), and tested which biotic and abiotic factors explain the reproductive success of the populations; and (iii) we evaluated the effects of generalization or specialization in the use of temporal (flowering phenology) and pollination (individual pollinator-plant interactions) resources for individual and population reproductive success. Floral traits and reproductive biology (nocturnal anthesis) of *T. laniflora* were associated to a specialized pollination system mediated by large bees with crepuscular foraging behavior. The spatially isolated populations were under different microclimatic conditions, and differed in their reproductive biology (onset and peaks of flowering and fruiting, floral biology (anthesis, longevity, and frequency of legitimate visits) and therefore, reproductive success. Abiotic factors, mainly temperature, drove the observed differences in phenological dates, while biotic factors related to plant-pollinator interactions directly influenced the production of mature fruits and differences on populations reproductive success. Finally, populations composed of more generalist individuals in their interactions with pollinators and more synchronic in their flowering activity (season, duration and intensity) showed greater reproductive success. At the individual level, more central and generalist plants in their interactions with the main pollinators were also more successful. Therefore, generalization and low specialization in the resources use, flowering time and pollinators played a key role in the conservation of plant-pollinator interactions of *Trembleya*, positively affecting the reproductive success of this species presenting a highly specialized pollination system.

Sumário

INTRODUÇÃO GERAL

Indivíduos coespecíficos utilizam de forma diferente os recursos a eles disponíveis no ambiente (eg. presas, nutrientes do solo, substrato, plantas hospedeiras), e essas variações intraespecíficas nos caracteres das espécies têm função fundamental em muitos processos ecológicos e evolutivos (Bolnick et al., 2003, Araújo et al., 2010, 2011; Bolnick et al., 2011; Dall et al., 2012; Wolf & Weissing, 2012; Zywiec et al., 2012; Tur et al., 2014). Estudos recentes têm atentado para a grande importância das variações intraespecíficas em caracteres das espécies na estruturação, estabilidade e funcionalidade das populações e comunidades de plantas (Jung et al., 2010; Violle et al., 2012; Siefert et al., 2015; Kuppler et al., 2016). Não é segredo que plantas são organismos altamente variáveis e que mudanças nos aspectos reprodutivos das espécies, como em sua fenologia de floração e frutificação (época, duração, sincronia e intensidade), são ecologicamente relevantes, tanto para as plantas, como para os animais com os quais elas interagem (Augspurger, 1981; Ollerton & Lack, 1992; Elzinga et al., 2007; Herrera, 1995a, 2009).

Padrões fenológicos de floração podem diferir entre indivíduos de uma mesma espécie (Herrera, 1988, 1992; Newstron et al., 1994; Sakai et al., 2005) e a fenologia vegetal é determinada por pressões seletivas de fatores bióticos (e.g. competição por polinizadores, dispersores e herbívoros), abióticos (temperatura, disponibilidade de água, luz e componentes minerais do solo), fisiológicos e filogenéticos (Wright & Van Shaik, 1994; Morellato et al., 2000; Borchert, 2002; Sakai, 2001; Elzinga et al., 2007; Staggmeier et al., 2010; Brito et al,. 2017). Respostas de floração e frutificação são influenciadas por fatores ambientais que, seguramente, sinalizam as estações climáticas e as condições mais favoráveis à reprodução das espécies (Rathcke & Lacey, 1985; Wright & Van Shaik, 1994; Morellato et al., 2000; Elzinga et al., 2007). Dessa maneira, o tempo (época e duração) de floração representa uma importante adaptação das plantas dependentes de animais para polinização, aos períodos de maior abundância do vetor de pólen; consequentemente, as interações planta-polinizador exercem forte pressão seletiva na fenologia reprodutiva destas espécies (Waser, 1983; Rathcke & Lacey, 1985; Van Schaik et al., 1993; Brito et al., 2017).

Adicionalmente, devido a um aumento potencial da atratividade das plantas para as espécies de polinizadores e, portanto, forte influência na efetividade das interações, variações

intraespecíficas em caracteres florais (como no horário de antese e longevidade floral) e nas respostas de floração (época, duração, sincronia e intensidade) podem determinar o sucesso reprodutivo de populações naturais de plantas, principalmente em espécies dependentes de vetores bióticos de pólen (Augspurger, 1981; Rathck, 1983; Rathcke & Lacey, 1985; Maroho, 2002; Dupont et al., 2014). A relação entre indivíduos de plantas e espécies de polinizadores pode ser descrita através de redes ecológicas (Fortuna et al., 2008) e o padrão de compartilhamento de polinizadores entre os indivíduos da rede representar o padrão de transferência de pólen, fecundação e fluxo gênico entre plantas (McDoanld, 2007; Gómez et al., 2011). O posicionamento dos indivíduos dentro das redes pode, desta maneira, estar relacionado a seu sucesso reprodutivo (Gómez and Perfeccti, 2012). Devido às interações planta-polinizador serem mediadas por características das espécies (eg. Stang et al., 2006; Dupont et al., 2011; Zywiec et al., 2012) e, ainda, por indivíduos serem os verdadeiros atores nas interações ecológicas (Tur et al., 2014), variações em caracteres florais no nível individual podem afetar a estrutura das redes planta-polinizador, interferindo assim na aptidão reprodutiva das populações vegetais (Gómez & Perfeccti, 2012; Kuppler et al., 2016). Desta maneira, para melhor compreendermos os fatores responsáveis pela estrutura, estabilidade e funcionalidade das populações e comunidades naturais, é fundamental entendermos também os efeitos da variação intraespecífica (intra e interpopulacional) de caracteres das plantas (morfológicos, fisiológicos e fenológicos) para as interações ecológicas das espécies (Bolnick et al., 2003; Gómez & Perfeccti, 2012; Kuppler et al., 2016).

A relação evolutiva entre polinizadores e caracteres florais das espécies é tópico historicamente discutido na biologia vegetal (Darwin, 1862; Faegri & Van der Pijl, 1979; Fenster et al., 2004; Ollerton et al., 2009; Rosas-Guerrero et al., 2014; Queiroz et al., 2015). A ocorrência das chamadas “síndromes de polinização” vem sendo testada, e a ideia da existência de uma relação direta entre, um conjunto de caracteres florais (como coloração, formato, tamanho, tempo e duração da antese, e tipo de recurso oferecido aos polinizadores) e o principal agente de polinização das espécies, tem sido geralmente confirmada (Faegri & Van der Pijl, 1979; Rosas-Guerrero et al., 2014; mas ver Ollerton et al., 2009). Os estudos sugerem que espécies de plantas são adaptadas a um grupo funcional de polinizadores que, por sua vez, exerce uma forte pressão seletiva nos caracteres florais (Fenster et al., 2004, Rosas-Guerrero et al., 2014).

Neste contexto, eventos de polinização mediados por abelhas (melitofilia) ocorrem, em geral, durante o dia, em espécies de plantas que também apresentam antese diurna (Faegri & Van der Pijl, 1979; Ollerton et al., 2009; Rosas-Guerrero et al., 2014). Entretanto, algumas espécies das famílias Apidae, Andrenidae, Halictidae e Colletidae forrageiam a procura dos recursos florais, principalmente pólen, em períodos do dia de pouca luminosidade, durante o amanhecer, o entardecer e, mais raramente, à noite (Janzen, 1968; Wolda & Roubik, 1986; Wcislo et al., 2004). Embora vários grupos de abelhas tenham desenvolvido este comportamento de forrageio noturno ou crepuscular, estudos de suas interações ecológicas, que avaliem a ocorrência de adaptações específicas das espécies vegetais à polinização pelas abelhas crepusculares, bem como a eficiência destas, como vetores de pólen, são ainda escassos e precisam, portanto, ser explorados (Hopkins et al., 2000; Somanathan & Borges, 2001; Wcislo et al., 2004; Franco & Gimenes, 2011; Cordeiro et al., 2017).

Em Melastomataceae, uma das famílias mais dominantes e diversas nos trópicos (Clausing & Renner, 2001; Goldenberg et al., 2012), a presença de flores de pólen (flores que oferecem apenas pólen como recurso para os polinizadores), heterostemia (ocorrência de diferentes estames na mesma flor) e deiscência poricida das anteras (antera tubular com poros apicais) são caracteres florais frequentes, sendo a deiscência uma característica diagnóstica da família e relacionada a um tipo especial de polinização, *buzz pollination* ou polinização por vibração, realizada exclusivamente por abelhas (Buchmann, 1983; Renner, 1989). Melastomataceae compõe uma das famílias mais ricas em espécies em áreas de campos rupestres, um rico complexo vegetacional composto por um mosaico de campos, associados a afloramentos de rocha, que ocorre predominantemente acima de 900m e até 2100 metros de altitude (Alves & Kolbek 2010; Silveira et al., 2016). Neste ambiente altamente rico em espécies (Silveira et al., 2016), Melastomataceae é representada principalmente por gêneros endêmicos como *Cambessedesia*, *Chaetostoma* e *Lavoisiera* (Giulietti et al., 1987; Alves & Kolbek, 2010).

Trembleya laniflora Cong. (Microlicieae) é uma espécie de Melastomataceae endêmica dos campos rupestres da porção sul da Cadeia do Espinhaço, no Estado de Minas Gerais (Sudeste do Brasil), apresentando uma distribuição restrita aos afloramentos de rocha (Martins, 1997). A espécie apresenta características florais que são raras na família, exibindo flores grandes e brancas, que destoam do padrão de coloração vibrante (frequentemente púrpura) observado

para a Tribo (Renner, 1989, Martins, 1997). Dessa maneira, considerando (i) as distintas características florais de *T. laniflora* e sua distribuição restrita aos afloramentos de rochas, (ii) as evidências da ocorrência das "síndromes de polinização" e, portanto, da existência de uma relação direta entre os caracteres florais das espécies e seus polinizadores efetivos principais, e ainda (iii) a importância de se avaliar os efeitos da variação intraespecífica (inter e intrapopulacional) em caracteres vegetais para as interações planta-polinizador e o sucesso reprodutivo de populações naturais de plantas, nós objetivamos neste trabalho:

1. Entender a importância ecológica e evolutiva dos distintos caracteres florais de *Trembleya laniflora* para sua polinização e sucesso reprodutivo nos afloramentos rochosos dos campos rupestres de Minas Gerais (Capítulo 1);
2. Avaliar a ocorrência de variações intraespecíficas na biologia floral, fenologia reprodutiva e polinização da espécie, testando para os fatores bióticos e abióticos que direcionam tais diferenças, e determinando a relevância destas variações para o sucesso reprodutivo de populações isoladas de *T. laniflora* (Capítulo 2);
3. Avaliar a variação intraespecífica na fenologia de floração e nas interações planta-polinizador em populações de *T. laniflora*, acessando como a especialização no nível individual e populacional se relacionam com o sucesso reprodutivo da espécie (Capítulo 3).

Nós pretendemos responder especificamente às seguintes questões:

1. As características florais (tamanho, coloração e tempo de abertura) de *Trembleya laniflora* indicam um comportamento de forrageio diferencial de seus polinizadores preditos (flores de pólen com polinização por vibração diurna realizada abelhas)? (Capítulo 1);
2. As características florais são consistentes com a hipótese da "síndrome de polinização" por melitofilia (*senso* Faegri and Van der Pijl, 1979)? (Capítulo 1);
3. *T. laniflora* é dependente dos polinizadores para reprodução e frutificação? (Capítulo 1);
4. Populações de *T. laniflora* localizadas em diferentes altitudes da Serra do Cipó, Minas Gerais, variam em sua fenologia (época, duração, sincronia), biologia floral (horário de antese e longevidade floral), e polinização (frequência de visitas e composição dos

polinizadores e outros visitantes florais), diferindo em seu sucesso reprodutivo? (Capítulo 2);

5. Quais fatores (bióticos e/ou abióticos) dirigem as variações intraespecíficas no sucesso reprodutivo das populações de *T. laniflora* naturalmente isoladas nos afloramentos de rochas? (Capítulo 2);
6. Indivíduos da espécie não especialistas em sua fenologia de floração (intensidade, duração e sincronia) e mais centrais nas redes de interação com as espécies de polinizadores, apresentam um maior sucesso reprodutivo, em comparação a indivíduos especialistas? (Capítulo 3).

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Capítulo 1

Crepuscular pollination and reproductive ecology of *Trembleya laniflora* (Melastomataceae), an endemic species in mountain rupestrian grasslands

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Chapter 1 - Crepuscular pollination and reproductive ecology of *Trembleya laniflora* (Melastomataceae), an endemic species in mountain rupestrian grasslands

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ABSTRACT

The "pollination syndrome" hypothesis predicts a direct relationship between a set of floral characters and the principal pollinating agent. The presence of flowers with poricidal anthers, heterostemony and pollen as the only reward are common traits in Melastomataceae species and are associated with buzz pollination by bees. *Trembleya laniflora* Cong. (Melastomataceae: Microlicieae) is an endemic species from *campo rupestre* tropical grassland, with large and white "pollen flowers" differing from the common purple-colored flowers of the Tribe. We examine the relationship between the distinct floral characteristics of *T. laniflora* and its pollination syndrome and reproduction ecology. We observed different individuals of *T. laniflora* randomly sampled in Serra do Cipó, Espinhaço Range, Minas Gerais, southeastern Brazil. We carried out observations on their reproductive phenology (31 individuals), flower biology (3), pollination (23), and tested the reproductive system (29). *Trembleya laniflora* presented a seasonal flowering pattern in the dry and post-dry seasons (May to October) and set fruits during the dry, post-dry and rainy seasons (June to December). Floral aperture occurred mainly during the night and the first hours of the morning, the period with the greatest availability of fresh flowers and with the highest visitation by *Xylocopa bimaculata*. Tests identified the species as non-apomictic, self-incompatible and dependent on large bees such as *Xylocopa*, *Bombus*, *Centris* and *Ptiloglossa* for pollination. *Trembleya laniflora* showed a specialized pollination system mediated by a restricted group of bees that perform crepuscular buzz pollination. Floral characteristics and reproductive biology of *T. laniflora* are likely adaptive responses to pollination by large bees foraging during the crepuscular hours. Our results support the "pollination syndrome" hypothesis, demonstrating a direct relationship between a set of floral characters and the principal pollinators of the species. Dependence on interbreeding may promote outcrossing within and among the endemic populations, naturally isolated on rocky outcrops.

Keywords: Floral traits, phenology, buzz pollination, reproductive system, germination, nocturnal bees

1. INTRODUCTION

The evolutionary relationship between pollinators and the floral traits of plant species is a topic historically described and discussed within plant biology (Darwin, 1862; Faegri and Van der Pijl, 1979; Fenster et al., 2004; Ollerton et al., 2009; Rosas-Guerrero et al., 2014; Queiroz et al., 2015). The occurrence of the so-called "pollination syndromes" has been tested, and the idea of a direct relationship between a set of floral characters (*e.g.* coloration, form, size, timing of anthesis and type of resource offered) and the principal pollinating agent generally persists (Faegri and Van der Pijl, 1979; Rosas-Guerrero et al., 2014; but see Ollerton et al., 2009). This finding suggests that plants are adapted to a specific functional group of pollinators that exert selective pressure on floral traits (Fenster et al., 2004, Rosas-Guerrero et al., 2014). However, in cases where a species presents floral characteristics matching more than one syndrome or has a wide diversity of pollinators (generalist species), the plant-pollinator interaction may not be so obvious (Herrera, 1987; Herrera, 2005; Ollerton et al., 2009; Queiroz et al., 2015).

Within this evolutionary context, plant species that have pollen as a primary resource to reward pollinators present floral traits often related to the protection of pollen, the selection of pollinating agents, and the precise deposition of pollen on the body of the pollinator (Buchmann, 1983; Pinheiro et al., 2014). In these so-called "pollen flowers" (Vogel, 1978), the presence of poricidal anther dehiscence (tubular anthers with apical pores) is common and is associated with heterostemony (the occurrence of different types of stamens in the same flower) (Vogel, 1978; Buchmann, 1983; Pinheiro et al., 2014). These characteristics are generally interpreted as strategies for solving the "pollen dilemma", in which the pollen grain fulfills a dual function: transporting the male gamete and being the resource supply for the flower visitors (Westerkamp, 1996; Lunau et al., 2011, 2015). The pollination of these pollen flowers through buzz pollination is performed exclusively by bees with the capability to vibrate their abdomen and extract the pollen from the anthers, which generally present poricidal dehiscence (Buchmann, 1983; Pinheiro et al., 2014).

In general, bee pollination (melittophily) occurs during the day, in plants that present flowers with a diurnal aperture (Faegri and Van der Pijl, 1979; Ollerton et al., 2009; Rosas-Guerrero et al., 2014). However, some species of Apidae, Andrenidae, Halictidae and Colletidae forage for floral resources (primarily pollen) in dim-light environments: at dawn,

dusk and occasionally at night (Janzen, 1968; Wolda and Roubik, 1986; Wcislo et al., 2004). The knowledge of the biology of these "crepuscular bees", their ecological interactions and role as pollinators are limited, probably because their dim-light foraging habits are often restricted to crepuscular periods and eventual nocturnal flights in the presence of moonlight (Burgett and Sukumalanand, 2000; Hopkins et al., 2000; Somanathan and Borges, 2001; Wcislo et al., 2004; Franco and Gimenes, 2011).

In Melastomataceae, one of the most dominant and diverse families in the tropics (Clausing and Renner, 2001; Goldenberg et al., 2012), poricidal anther dehiscence and heterostemony are frequent traits; indeed, poricidal anthers are a diagnostic characteristic of the family and associated with buzz pollination (Buchmann, 1983; Renner, 1989). In Brazil, this family figures among the most species-rich in the Atlantic forest, cerrado and *campo rupestre* (Giulietti et al., 1987; Mendonça et al., 2008). Neotropical bee species with vibratory capacity in Brazil belong to the families Apidae (e.g. Centridini, Bombini, Xylocopini, Meliponini), Halictidae (Augochlorini), Colletidae (e.g. Colletinae) and Andrenidae (Oxaeini) (Nunes-Silva et al., 2010).

Species of *Trembleya* (Melastomataceae), endemic to Brazil, present pollen flowers with poricidal anthers and heterostemony. The diplostemonous androecium is composed of distinct stamens arranged in two circles (antesepalous and antepetalous) (Martins, 1997). Ninety percent of the species of this genus are distributed in the *campo rupestre* of Minas Gerais (Martins, 1997) and are well represented in this heterogeneous vegetation dominating the Espinhaço Range above 900 m altitude (Conceição and Pirani., 2005, 2007; Rapini et al., 2008; Silveira et al., 2016). *Trembleya laniflora* Cong. (Melastomataceae: Microlicieae) is a species endemic to the *campo rupestre* in the south of the Espinhaço range and presents floral characteristics that are rare in the family (Renner, 1989): large flowers of white coloration, which differs from the vibrant, often purple color pattern found in the Tribe. Purple-magenta flowers are common in species of *Trembleya*, while entirely white petals are described only for *T. laniflora*, *T. debilis* and *T. hirsutissima* (Martins, 1997).

The study of plant reproductive biology, when associated with phenology (Ollerton and Lack, 1992; Ollerton and Dafni, 2005), the reproductive system (Zapata and Arroyo, 1978; Santos et al., 2012), pollination ecology (Brito and Sazima, 2012), and seed dispersal (Levin et al., 2003) and germination (Rodrigues and Silveira, 2013), contributes to a better

understanding of the ecology and evolution of natural populations (Pombal and Morellato, 2000; Silveira et al., 2015). Therefore, by integrating the above information, we aimed to understand the ecological and evolutionary importance of the distinct floral characters of *Trembleya laniflora* for its successful reproduction on the rocky outcrops of *campo rupestre*. More specifically, we sought to answer the following questions: i) Do the floral characteristics (size, color, and timing of aperture) of *T. laniflora* indicate a differential foraging behavior of its predicted pollinators (pollen flowers with diurnal buzz pollination by bees)? ii) Are the flower traits consistent with the "pollination syndrome" hypothesis of melittophily (*senso* Faegri and Van der Pijl, 1979)? iii) Does *T. laniflora* depend on pollinators for reproduction and fruit set? We expect that the presence of large, white flowers of *T. laniflora* is an adaptive response to pollination by large crepuscular bees. We also expect that *T. laniflora* dependence on interbreeding contributes to increase outcrossing and maintain the diversity of this species, given its distribution restricted to *campo rupestre* rocky outcrops. We discuss the "pollination syndrome" hypothesis and the ecological and evolutionary importance of pollination by crepuscular bees.

2. MATERIAL AND METHODS

2.1. Study area – The study was conducted from January 2014 to December 2015 in an area of *campo rupestre*, locally referred to as *Quadrante 16* (Q16), situated at 1303 m altitude (19°17′42.0″S; 43°35′31.2″W) and within the environmental protection area of Morro da Pedreira (APA Morro da Pedreira) at Serra do Cipó (SC), south of the Espinhaço Range, Minas Gerais, southeastern Brazil (Rocha et al., 2016). The climate at the SC region is classified as Cwp type (Köppen, 1931)—mesothermal, with well-defined dry and wet seasons organized in four distinguishable periods: dry season (May to September); post-dry season (October); rainy season (November to January); and post-rainy season (February to April) (Madeira and Fernandes, 1999; Silveira et al., 2016). The average annual mean temperature and total precipitation are 21.2 °C and 1,622 mm, respectively (Madeira and Fernandes, 1999). The vegetation of the study area is classified as *campo rupestre sensu stricto* (Silveira et al., 2016), on quartzitic and sandy soils, and is composed of a vegetation mosaic dominated by meadows (sandy, rocky and wet grasslands dominated by Poaceae) and rocky outcrops associated with herbs, sub-shrubby and shrubby species mostly from the families

Melastomataceae, Velloziaceae, Malpighiaceae and Euphorbiaceae (Giulietti et al., 1987; Silveira et al., 2016).

2.2. Study species – *Trembleya laniflora* Cong. (Melastomataceae) is a shrubby species endemic to the *campo rupestre* of Minas Gerais, with an occurrence restricted to rocky outcrops (Martins, 1997). Flowers of the species are pentamerous (frequently, individuals present some tetramerous and/or hexamerous flowers), large (≥ 4.5 cm), showy and white-colored, with some individuals presenting small pink spots on the petals (Figure 1) (Martins, 1997). The diplostemonous androecium presents heterostemony, with 10 glabrous stamens that are distributed in antepetalous and antesepalous circles and are distinguished by the size and coloration of the thecae (Martins, 1997). The fruits are dry, capsular and dehiscent, with numerous (from 400 to 850 seeds per fruit; personal observation), diminute (up to 0.57 mm) self-dispersed seeds (Martins, 1997).

We separated the two circular groups of stamens in the androecium according to their putative functions (Renner, 1989; Martins, 1997; Ollerton and Dafni, 2005): the stamens in the antepetalous circle (short, with yellow-colored thecae) are those responsible for the production of food pollen (hereafter feeding anthers); and the stamens in the antesepalous circle (long, with purple-colored thecae and presence of the connective) are those responsible for the production of pollen for pollination (hereafter pollination anthers) (Figure 1). Thus, we defined the occurrence of two types of pollen: one from the feeding anthers and the other from pollination anthers.

2.3. Flower biology – To study the floral biology (timing of opening and floral longevity), we tagged 40 pre-anthesis floral buds from three *Trembleya laniflora* individuals randomly chosen from throughout the area (Q16). The timing of flower opening and closing, as well as floral longevity, were determined by monitoring the tagged floral buds from the beginning of anthesis until the wilting and closing of the flowers. We observed the floral bud and flower development stages during four consecutive days, from dawn until dusk (05:30–18:30 h), performing hourly surveys, except during the dawn (05:30–06:30 h), when the surveys occurred every 30 minutes. On the fourth day of observation, we made only four surveys throughout the day (06:00 h; 07:00 h; 12:00 h and 18:20 h), since most flowers were already senescent. The stigmatic receptivity was tested immediately after flower opening, using

hydrogen peroxide (20% H_2O_2) on 22 fresh flowers randomly collected from individuals randomly chosen inside the study area (Dafni et al., 2005).

2.3.1. Pollen viability: To confirm the existence of two pollen types, we tested the viability of the pollen from the feeding and pollination anthers. Pre-anthesis floral buds from seven *T. laniflora* individuals were first isolated in organza mesh; after anthesis, 35 flowers were collected and fixed in a formaldehyde solution (FAA 80%). From each flower, we extracted and separately macerated six anthers (three feeding and three pollination anthers). The obtained samples were diluted in ethyl alcohol (80%), stained with acetic carmine (2%) and analyzed in an electronic light microscope (40X objective) (Radford et al., 1974). We counted 214 pollen grains derived from feeding anthers and 231 from pollination anthers, and we quantified the number of viable (colored grains) and non-viable (not colored) pollen grains in each sample. To compare the viability of the pollen grains, we performed a two-sample proportion test.

2.4. Phenology – For the phenological monitoring, we randomly sampled and tagged 31 reproductive *T. laniflora* individuals from a 100 m x 100 m (1 ha) plot inside the study area (Q16). Phenological observations were conducted monthly from January to December 2014; we counted the number of reproductive structures (floral buds, flowers, immature fruits and mature fruits) on five tagged branches of each individual (Ollerton and Dafni et al., 2005; Athayde and Morellato, 2014). During the flowering peak (July–August) of 2014, phenological observations were conducted every 15 days. Previous phenological observations carried out in 2012 and 2013 confirmed the annual flowering pattern of *T. laniflora*. However, due to fires in September 2014, we lost 15 out of 31 sampled individuals; therefore, the phenological observations from September to December 2014 refer to only 16 individuals.

2.5. Reproductive systems – To examine the reproductive system of *T. laniflora* and test the importance of the pollinators and both pollen types for reproduction, we performed controlled field tests of pollination on 29 *T. laniflora* individuals randomly selected inside the study area (Q16) (Zapata and Arroyo, 1978; Dafni et al., 2005), as well as controlled lab tests of germination and presence/viability of embryos (Zapata and Arroyo, 1978). The controlled pollination tests checked for the occurrence of **1) *cross-pollination (CP)*** — after anthesis of previously isolated buds on 20 *T. laniflora* individuals, 146 flowers were emasculated and

pollinated manually with pollen collected from nearby individuals. We pollinated 74 flowers with pollen from the pollination anthers (*CP_poll*) and 72 flowers with pollen from the feeding anthers (*CP_feed*); **2) *self-pollination (SP)*** — after the anthesis of the bagged buds, 150 flowers of 12 individuals were emasculated and manually pollinated with their own pollen. We treated 77 flowers with pollen from pollination anthers (*SP_poll*) and 73 with pollen from the feeding anthers (*SP_feed*); **3) *autonomous self-pollination (AS)*** — on 10 individuals, we isolated 86 floral buds in small organza bags and monitored their phenology until the principal fruiting period; **4) *apomixis (AP)*** — we emasculated 79 pre-anthesis floral buds from thirteen different individuals, isolated the emasculated buds with organza bags and monitored them until the fruiting period; **5) *control (CN)*** — we monitored the reproductive phenology of 245 previously tagged floral buds on nine treated individuals. The fruits resulting from the various tests were collected, and the seeds from the 20 fruits in each treatment were counted and weighed on a precision balance (Model AR2140, e = 1 mg, d = 0.1 mg). To test for difference among the treatments, we quantified i) the number of fruits formed; ii) the number of seeds formed per fruit (N = 20 fruits per treatment); and iii) the seed weight (total weight/number of seeds; N = 20 fruits per treatment).

The reproductive system was assessed based on the conversion rates (flower/fruit), the reproductive efficacy (RE), the self-incompatibility index (ISI) and the autonomous self-pollination index (IAS) (Zapata and Arroyo, 1978), and the presence and viability of the embryo after the germination experiments (Brasil, 2009). RE of each treatment was calculated by dividing the conversion rate (flower/fruit) of the treatment by the conversion rate of the control (SP/CN and CP/CN). RE values close to 0 indicate low reproductive efficacy, while values close or equal to 1 indicate high reproductive efficacy. ISI was calculated by dividing the conversion rates for the self-pollination treatments by those for the cross-pollination treatments (SP/CP). Self-compatibility is indicated by ISI = 1, incomplete self-compatibility by $0 < ISI < 1$, and self-incompatibility by ISI = 0. IAS was obtained through dividing the conversion rate of the autonomous self-pollination treatment by that of the control (AS/CN); an IAS value = 1 indicates the occurrence of autonomous self-pollination, and IAS = 0 indicates the absence of autonomous self-pollination (Zapata and Arroyo, 1978).

2.5.1. Seed germination test: All seeds produced by fruits following the cross-pollination, self-pollination and control tests were placed for germination in Petri dishes covered by filter

paper moistened with distilled water, exposed to temperatures of 20–30 °C and a 24-h photoperiod (Rodrigues and Silveira, 2013). For each treatment (*CP_poll*, *CP_feed*, *SP_poll*, *SP_feed*, and *control*), we used six samples of 25 seeds, total 150 seeds per treatment. Autonomous self-pollination and apomixis treatments did not set fruit or viable seed (see Results).

We inspected the seeds every 48 hours, using radicle emergence (visible to the naked eye) as the criterion of germination (Labouriau, 1983). After a minimum of 30 days following initiation of the germination experiments, all non-germinated seeds were submitted to the tetrazolium test and examined for the presence and viability (coloration) of the embryo. We placed the seeds in a tetrazolium solution (1%), conditioned to a temperature of 30 ºC, for a minimum of 24 hours (minimum time for coloration of tetrazolium solution); we then cut the seeds and observed them in a 4.5x magnifying glass loupe (Brasil, 2009).

2.6. Pollination ecology – The flower visitors and their foraging behavior (resource sought and time of visit) were observed in July and August 2014 (80 hours of observation) and in July 2015 (43 hours of observation). In 2014, we observed visitations to 23 *T. laniflora* individuals that had been sampled for phenological observations and had blossomed during the flowering peak of the species (July and August). We randomly performed 10-minute surveys in each individual between 05.30 h and 18.30 h (dawn to dusk) each day (Herrera, 1995). In 2015, over four consecutive days, between 05.30 h and 18.30 h, we made focal observations of four *T. laniflora* individuals chosen from the sample area (Q16) (Dafni et al., 2005). The dawn and dusk periods were defined taken into consideration of the short days during the dry season and sunrise after 06.00 h.

In 2014, we aimed to assess the diversity of flower visitors and pollinators, mainly by observing the behavior of insects on the flowers (Herrera, 1995; Dafni et al., 2005). In 2015, however, we focused on identifying the flower visitors and the effective pollinators of *T. laniflora*, classifying the latter as principal or secondary according to their collection and foraging behavior, as well as assessing the frequency of visitation (Inouye et al., 1994; Rosas-Guerrero et al., 2014; Dafni et al., 2005). Among the effective pollinators, we considered as main pollinators the large bee species ($\geq$ 2.5 cm) that were capable of vibrating both types of anthers concurrently and contacted the reproductive flower parts 10 times or more (N $\geq$ 10

visits); we considered as secondary pollinators the large bees (≥ 2.5 cm) that possessed vibration capability and contacted the reproductive parts less than 10 times (N ≤ 10 visits). Small bees (≤ 1.5 cm) that were not capable of vibrating both types of anthers concurrently, pollen thefts and other insects that visited the flowers but did not contact the stigma were considered flower visitors.

Specimens not identified in the field were collected and/or filmed with a SONY HDR-SR11 video camera and then deposited in the Entomological Collection of the Department of Zoology, IB, UNESP, campus of Rio Claro, SP. The data collected in 2014 (the year with the most hours of observation) were used to analyze visitation over time. The 2015 data from the focal observations were used only to compose a list of the *T. laniflora* flower visitors and effective pollinators (main and secondary), enabling the detection of less frequent and/or rare species (Dafni, 1992). Due to the morphological similarity of *Eulaema nigrita* and *Bombus pauloensis* (large bees considered here as effective pollinators) and the difficulty of distinguishing them in the field, we combined their data for the visitation analysis.

2.7. Data Analyses – The reproductive phenology pattern of the species was tested through circular statistics (Morellato et al., 2000), using the data (dates of flowering and fruiting) obtained between January and December 2014. The Rayleigh (z) test which measures the significance of the average angle (Zar, 1996) was applied to detect the occurrence of a significant seasonal pattern in the phenophases (Morellato et al. 2000).

We applied a two-sample proportion test to compare the conversion rates (flower/fruit) between the controlled pollination experiments and each treatment as well as between the treatments. Differences between each treatment in the number of seeds produced per fruit were tested through the Kruskal-Wallis test, while differences in the weights of the seeds from fruits formed in the various treatments were tested through the ANOVA and a posterior Tukey test (Zar, 1996). We performed the circular analyses in Oriana 4 and the other the analyses in the software R version 3.1.3 (R Development Core Team, 2011).

3. RESULTS

3.1. Flower biology

Trembleya laniflora presented a mainly nocturnal and/or crepuscular floral opening. Of the 40 tagged buds, 18 (45%) opened during the night (between 18.30 h and 05.30 h), and 13 (32.5%) opened during sunrise (from 05.30 h to 06.30 h). The majority of flowers (90%) were available to pollinators in the first hours of the morning, and we did not observe floral opening during the afternoon (Figure 2).

The flowers remained open for a minimum of two days. Of the 38 tagged flowers, 23 (60.5%) remained open for three days, 10 flowers (26.5%) for two days, and five flowers (13.2%) for up to four days. The stigma was receptive from the beginning of the anthesis (19 receptive/22 tested). Pollen from both of the anthers presented high viability, with a proportion equal to 96% for the two types of pollen.

3.2. Phenology

Trembleya laniflora flowered during the dry and post-dry seasons (May–October) and fruited throughout the dry and post-dry seasons and the beginning of the rainy season (June–December) (Figure 3). Flowering peaked during the dry season, in June (floral bud) and July (anthesis), while fruiting peaked at the end of the dry season (August; immature fruit) and post-dry season (October; mature fruit) (Figure 3 and Table 1, supplementary material). Reproduction presented a significant and highly seasonal pattern ($r \geq 0.96$; Table 1, supplementary material). We observed the following maximum monthly productions (production peaks): 43,047 floral buds; 38,619 flowers; 73,888 immature fruits; and 10,000 mature fruits (Figure 3).

3.3. Reproductive system

3.3.1. Controlled Pollination Test: *Trembleya laniflora* produced fruits and seeds in the cross-pollination and self-pollination tests, independent of the pollen type (treatment), and showed high values of fruit set (≥ 0.68) in both tests (Table 1). ISI values were equal to 1 in both of the induced self-pollination treatments, indicating compatibility independent of the pollen origin. The formation of fruits by apomixis was not significant (Fruit set $_{AP} = 0.03$),

and we found no evidence of autonomous self-pollination (IAS = 0). The production of fruits by cross-pollination and by induced self-pollination was effective (RE > 0.9). Rates of flower/fruit conversion (fruit set) obtained for cross-pollination treatments did not differ from the rates for the self-pollination treatments (CP_Poll x SP_Poll, χ^2 = 0.1,p = 0.74 and CP_Feed x SP_Feed χ^2 = 0.3, p = 0.54). We did not observe significant differences between the conversion rates for the treatments of each test (Table 1). We found significant differences when comparing the conversion rate between the control and the autonomous self-pollination and apomixis. Differences were also significant between CP and SP treatments with AS (Table 1).

Regarding seed production, we found significant differences between the number of seeds produced per fruit (K = 10.42, p = 0.03; Figure 1, supplementary material) in the experiments (CP x SP). Fruits resulting from the cross-pollination treatments (CP_Poll and CP_Feed) produced more seeds than those obtained from the self-pollination treatments (SP_Poll and SP_Feed) and from the control (CN) (see median values, Figure 1, supplementary material). We found no significant differences in seed weight among treatments (F = 1.34, p = 0.26 ANOVA) (Figure 2, supplementary material).

3.3.2. Seed germination: We observed germination in only nine seeds, all of which resulted from cross-pollination treatments with pollen from the feeding anthers (Figure 4). *T. laniflora* seeds presented embryos only in the interbreeding experiments (CP_Feed and CP_Poll). However, we did not find viable stained embryos (colored) after immersion of the seeds in the tetrazolium solution. For all treatments, we found a high proportion (69–99%) of embryoless seeds (Figure 4).

3.4. Pollination Ecology

We observed 29 insect species visiting the *T. laniflora* flowers (Table 2, supplementary material), and bees were the most common flower visitors (Figures 5; Table 2, supplementary material). Among the species found, 13 were considered as pollinators (three main and 10 secondary); the others included four flower visitors and 12 pollen thefts (Table 2, supplementary material). We counted 254 visits to the *T. laniflora* flowers: 117 (46%) by pollinators and the remaining 137 (54%) by flower visitors. The pollination events occurred

mainly during the short crepuscular periods (42%) at dawn (from 06:00 h to 07:00 h) and dusk (from 17:30 h to 18:00 h) and during the morning period (from 08:00 h to 11:00 h) (40%), with peak pollination (N = 24) occurring in the morning twilight between 06:00 h and 06:30 h (Figure 5A). Flower visitor frequency was highest from 09:00 h to 14:30 h (N = 101), with peak activity from 10:30 h to 11:00 h (N = 20). *T. laniflora* generally presented a low frequency of flower visitors during the day (< 10%; Figure 5B).

Among the main pollinators, *Xylocopa* (*Dasyxylocopa*) *bimaculata* was the most effective, contributing 34% (N = 40) of all pollination events, followed by *Xylocopa* sp1, with 38 visits to the flowers (32%) and *Xylocopa* sp4 with 12 visits (10%). The other 14% of the visits were shared among *Eulaema nigrita*/*Bombus pauloensis* (N=15), *Centris* sp1 (N=5) and *Oxaea* sp (N=4), all secondary pollinators (Figure 5A). Visits by *X. bimaculata* and *Xylocopa* sp4 were mostly or exclusively crepuscular, while *Xylocopa* sp1, *Eulaema nigrita*/*Bombus pauloensis* and *Centris* sp1 visits were primarily in the morning (08:00 h to 11:30 h) (Figure 5A). *Oxaea* sp was the only species of pollinator active in the afternoon (Figure 5A). Among the species classified only as flower visitors, *Melipona trinofaciata* was the most frequent, with 78% of the visits (107), followed by *Augochloropsis* sp4 (7) and *Augochloropsis* sp5 (5) (Figure 5B). Due to their small body size (≤ 1.5 cm) and behavior on the flowers, these three species were considered as pollen thefts or cheaters, not contributing to the reproduction of *T. laniflora*.

4. DISCUSSION

The large, white flowers of *T. laniflora* did not conform to the expected characteristics of classic melittophily syndrome (flowers of often vibrant color and diurnal floral aperture; Faegri and Van der Pijl, 1979; Ollerton et al., 2009; Rosas-Guerrero et al., 2014). Instead, the *T. laniflora* flowers were open during the dawn and dusk hours and were mainly pollinated by bees with crepuscular foraging behavior. In Melastomataceae, white, cream or discrete flowers are generally small and typically associated with apomictic species that do not depend on pollination services to set fruits (Goldenberg and Shepherd, 1998, Brito et al., unpublished data). Apomixis is a common feature in the family, occurring in up to 88% of the species in the tribe Miconieae (Renner, 1989; Goldenberg and Shepherd, 1998; Santos et al., 2012). However, we did not consider *T. laniflora* as apomictic, since no fruits were formed after

emasculation. The presence of large, white flowers was associated with anthesis at dawn and dusk and the pollination by crepuscular bees.

The absence of apomixis may be associated with the endemism and restricted occurrence of *T. laniflora* on rocky outcrops of *campo rupestre* in Minas Gerais (Martins, 1997; Santos et al., 2012). The relationship between the reproductive system of Melastomataceae and the geographic distribution of the species is a topic already described in the literature (Goldenberg and Shepherd, 1998; Andrade et al., 2007; Santos et al., 2012). In general, apomictic species present a wider geographic distribution than those dependent on sexual reproduction, and the latter are frequently endemic or have restricted distribution (Goldenberg and Shepherd, 1998; Santos et al., 2012). The high pollen viability, independent of the type of anther (feeding or pollination), observed in *T. laniflora* is also evidence of the species' investment in interbreeding (Goldenberg and Shepherd, 1998; Santos, 2008).

The absence of germination and of viable embryos in the seeds from self-pollination experiments indicate that *T. laniflora* is self-incompatible and dependent on cross-pollination, even though the ISI values (equal to 1) have suggested self-compatibility, and the flower/fruit conversion rates of SP and CP treatments did not differ. The lack of fruit set or seeds by autonomous self-pollination reinforces the classification of *T. laniflora* as self-incompatible and dependent on interbreeding mediated by bees. The poricidal anthers and the dependence on vibration to release pollen prevent the occurrence of autogamy (self-pollination by pollen from the same flower) and favor the predominance of allogamy (interbreeding) in *T. laniflora*. The absence of autonomous self-pollination is a common characteristic of Melastomataceae, and it has been associated with the position of the anthers in relation to the stigma during anthesis (anthers stay coiled beneath the stigma from bud stage until flower opening) and with the necessity of vibration (buzz pollination) to remove pollen from the anthers (Renner, 1989; Goldenberg and Shepherd, 1998). The dependence on pollinator-mediated interbreeding may promote genetic variability within the populations of endemic *T. laniflora* that occur naturally isolated on rocky outcrops, a topic to be explored in future research.

Trembleya laniflora was pollinated exclusively by large bees in the genera *Xylocopa*, *Bombus*, *Eulaena* and *Centris*, all capable of vibrating both circles of the poricidal anthers. Pollination occurred mainly during the highest availability of fresh, recently opened flowers

in the crepuscular hours of the day. The crepuscular bees *Xylocopa* (Dasyxylocopa) *bimaculata* and *Xylocopa* sp4 contributed to 44% (N = 52) of all observed pollination events, mostly during short periods of dim sunlight. Therefore, large bees with vibratory behavior and crepuscular foraging activity are the most effective pollinators of *T. laniflora*. The timing of floral opening and pollination were associated with the foraging behavior of those bees during the crepuscular hours of reduced light, an activity pattern known for some species of the families Apidae and Collectidae, especially from the genera *Xylocopa*, *Centris* and *Ptiloglossa* (Janzen, 1968; Wolda and Roubik, 1986).

Pollination by vibration (buzz pollination) is common in Melastomataceae (Renner, 1989; Harter et al., 2002, Brito and Sazima, 2012), and the evolutionary history of the family is associated with the vibratory behavior of bees (Vogel, 1978; Buchmann, 1983; Renner, 1989). Visits in periods of low light, during the minutes that precede the dawn, are reported for the species in the family (Frankie, 1976; Renner, 1989); nevertheless, the pollinators and their behavior are poorly described. With respect to the biology of bees with crepuscular foraging activity on flowers, knowledge is still scarce (Hopkins et al., 2000; Wcislo et al., 2004), and we are just starting to discover the importance of crepuscular vibratory bees for pollination.

The observed flowering and fruiting patterns have also been considered as adaptations of the species to effective pollination and to seed dispersal (Salazar et al., 2011; Silveira et al., 2012, Brito et al. unpublished data). *Trembleya laniflora* presents a large production of flowers and demonstrated highly seasonal flowering during the dry season and seed dispersal at the beginning of the rainy season. Flowering during the dry season is associated with the presence of pollinators and the highest rate of pollination by bees (Janzen, 1968). Reproduction during the dry season is described for sub-shrubby species of the family Melastomataceae of the sandy- and rocky-soil meadows of Serra do Cipó (Le Stradic, 2012). The fruiting period corroborated previous descriptions of the species as presenting capsular, dehiscent fruits that open for the autochorous dispersal of seeds at the beginning of the rainy season (Silveira et al., 2012; Rodrigues and Silveira, 2013). In this period, the temperature and light conditions are the most adequate for seed germination and seedling development (Madeira and Fernandes, 1999; Silveira et al., 2012, Rodrigues and Silveira, 2013).

The high production of flowers, coupled with the differing quantities of immature and mature fruits produced, high percentages of embryoless seeds, and low germinability may be related to the frequent occurrence of abortions in Melastamataceae (Simão et al., 2007; Rodrigues and Silveira, 2013; Dayrell et al., 2016). Embryoless and non-viable seeds are a phylogenetically determined characteristic, frequent in Melastomataceae species of *campo rupestre* (Dayrell et al., 2016). Both characters are common in *T. laniflora* (Rodrigues and Silveira, 2013), and the occurrence of abortions in the family is related to occasional self-pollination events and malformation of the seeds, an outcome of nutritional deficiencies during development (Renner, 1989; Simão et al., 2007; Brito and Sazima, 2012; Rodrigues and Silveira, 2013). The *campo rupestre* presents highly P-deficient soils (Oliveira et al., 2015); therefore, the occurrence of embryoless and non-viable seeds in *T. laniflora* may also be associated with a P-limitation and consequent low investment in sexual reproduction and seed dispersal (Hopper, 2009; Fujita et al. 2014).

An additional hypothesis for the high investment in the formation of fruits with non-viable seeds would be the occurrence of inbreeding in the populations (Holmes et al., 2008; Dayrell et al., 2016). The dependence on interbreeding mediated by pollen vectors, coupled with seed dispersal over short distances by autochorous dispersal (van der Pilj, 1982) and the endemism of the species to rocky outcrops that are generally spatially isolated, may lead to endogamy. Thus, we suggest that the low production of viable embryos and the occurrence of frequent abortions are also consequences of a possible kinship mating between individuals and low genetic variability of the populations (Dubash and Fenster, 2000; Oostermeijer et al., 2003; Holmes et al., 2008).

Here, we demonstrated that *T. laniflora* presents a specialized buzz pollination system mediated by large crepuscular bees. We confirmed that the distinct floral characters (large, white-colored pollen flowers) and reproductive biology (nocturnal anthesis, self-incompatibility, large production of flowers) of *T. laniflora* are coupled with pollination by crepuscular bees. Therefore, although they differ from the classic pattern expected of melittophily species of Melastomataceae, the morphological characters (size and coloration) and flower biology (timing of anthesis) of *T. laniflora* are related to the behavior of its most effective pollinators. They are also consistent with the general "pollination syndromes"

hypothesis of a direct relationship between a set of floral characters and the principal pollinating agent of the species (Faegri and Van der Pijl, 1979; Ollerton et al., 2009; Rosas-Guerrero et al., 2014).

The self-incompatibility of *T. laniflora* was confirmed only after the seed germination tests, and it is considered as a mechanism to avoid inbreeding and favour the occurrence of interbreeding in naturally isolated populations. The combined observations of flower biology, phenology, reproductive system, germination and pollination revealed the reproductive biology of this endemic species. Similar integrative studies of reproductive biology will improve our understanding of the ecology and evolution of native species and the dynamics of their populations. Such studies are especially relevant when considering endemic species that grow isolated in stressful environments such as the *campo rupestre* (Silveira et al., 2015).

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7. TABLE AND FIGURES

7.1. Tables

Table 1: Results of reproductive system experiments Fruit set, Index of self-incompatibility (ISI), Index of autonomous self-incompatibility (IAS) and Reproductive efficacy (RE) of *Trembleya laniflora* (Melastomataceae), Serra do Cipó, Minas Gerais, Brazil. Parentheses indicate the number of treated buds; Fruit set = conversion rate flower/fruit; (Fruit/Flower) = number of production fruits/number of flowers treated. Different letters indicate significant differences after two-sample tests (p<0.05). ISI, IAS and RE range from 0 to 1.

Treatment	Fruit set	ISI	IAS	ER
Control (245)	0.71 (173/245) [a]			
Cross pollination_pollination pollen (74)	0.73 (54/74) [a]			1.00
Cross pollination_ feed pollen (72)	0.68 (49/72) [a]			0.96
Self pollination_ pollination pollen (77)	0.76 (59/77) [a]	1.00		1.00
Self pollination_ feed pollen (73)	0.74 (54/73) [a]	1.00		1.00
Autonomous self-pollination (86)	0.00 (0/86) [b]		0.0	
Apomixis (79)	0.03 (2/79) [b]			0.04

7.2. Figures

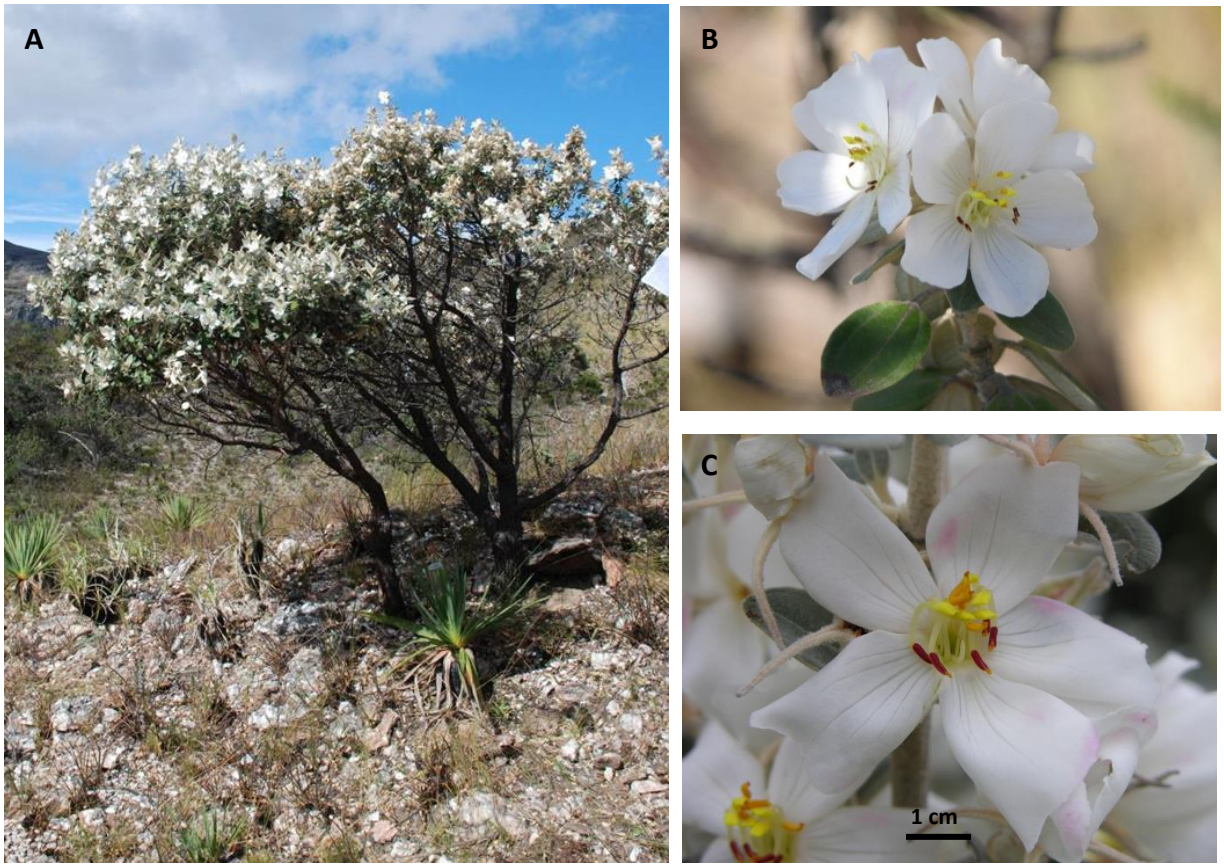

Fig.1. General aspects of *Trembleya laniflora* (Melastomataceae). Individuals of *T. laniflora* in the rocky outcrop of *campo rupestre* at Serra do Cipó (Minas Gerais, Brazil) (A); detail of the inflorescence (B); and a single flower with some pink spots (C).

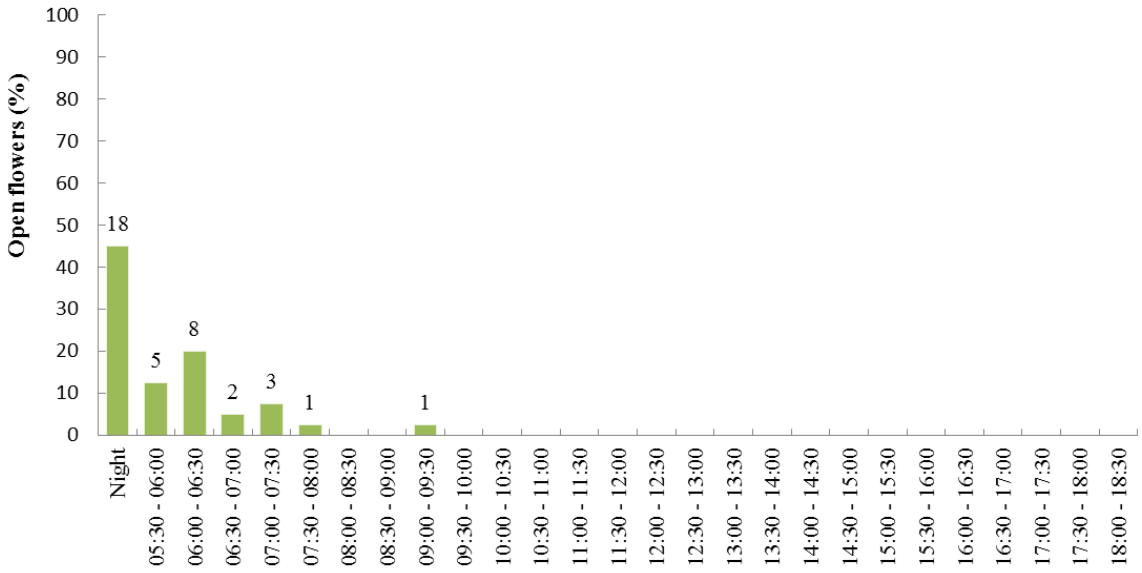

Fig.2. Timing of floral opening of *Trembleya laniflora* (Melastomataceae) at Serra do Cipó (Minas Gerais, Brazil). Percentage (bars) and respective numbers of flowers that opened at the different observation hours (N = 40 buds). Night represents the interval from 18:30 to 5:30 h between the present and previous days' observations.

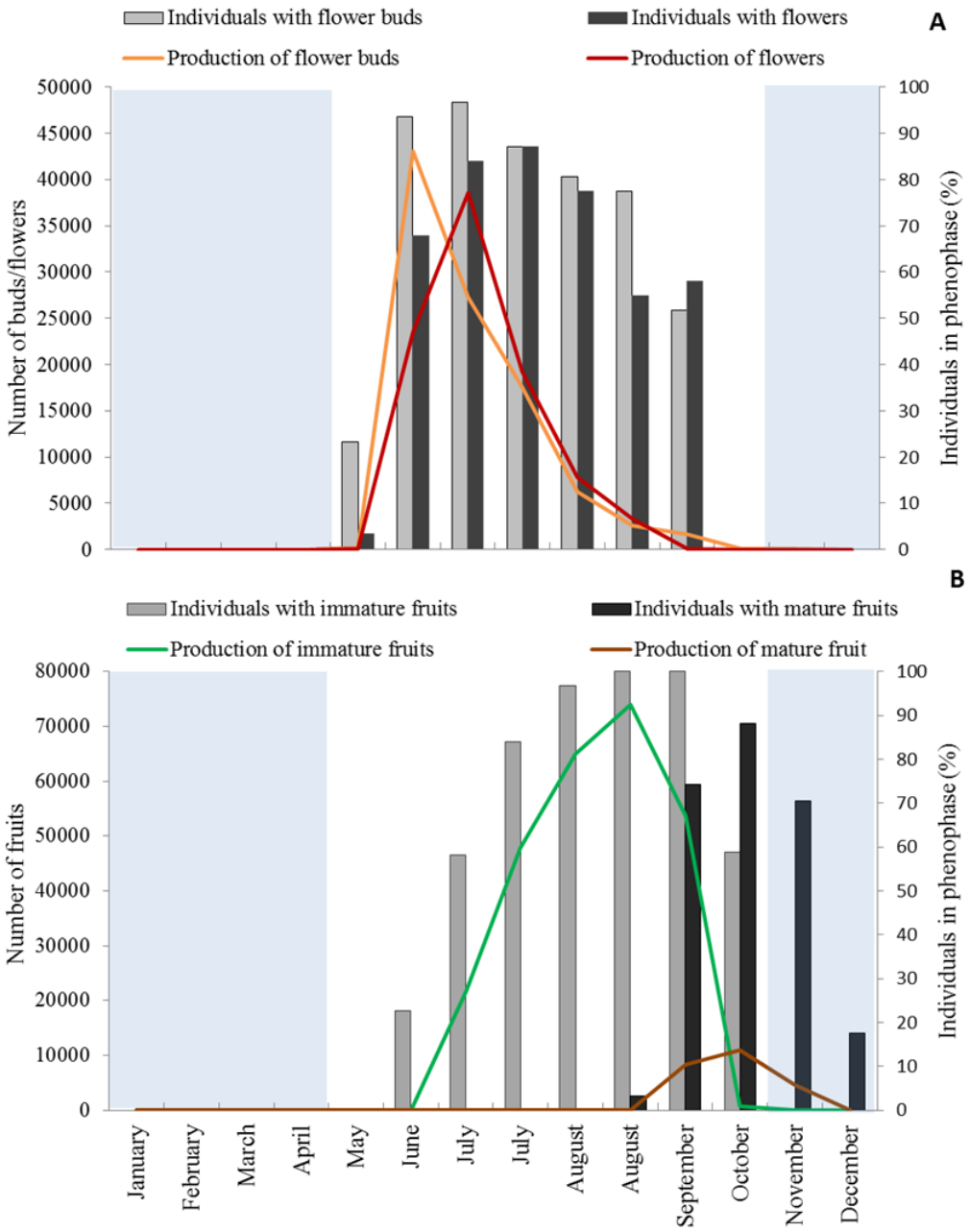

Fig.3. Reproductive phenology of *Trembleya laniflora* (Melastomataceae), in *campo rupestre* at Serra do Cipó (Minas Gerais, Brazil) during 2014, per number of reproductive structures (lines) and percent of individuals (bars). Monthly total production of (A) floral buds (orange line), flowers (red line), (B) immature fruits (green line) and mature fruits (brown line). Percentage of individuals with floral buds and immature fruits (grey bars in A and B, respectively) and the percentage of individuals with flowers and mature fruits (white bars in A and B, respectively), by month of phenological observation. The shaded area represents the rainy season.

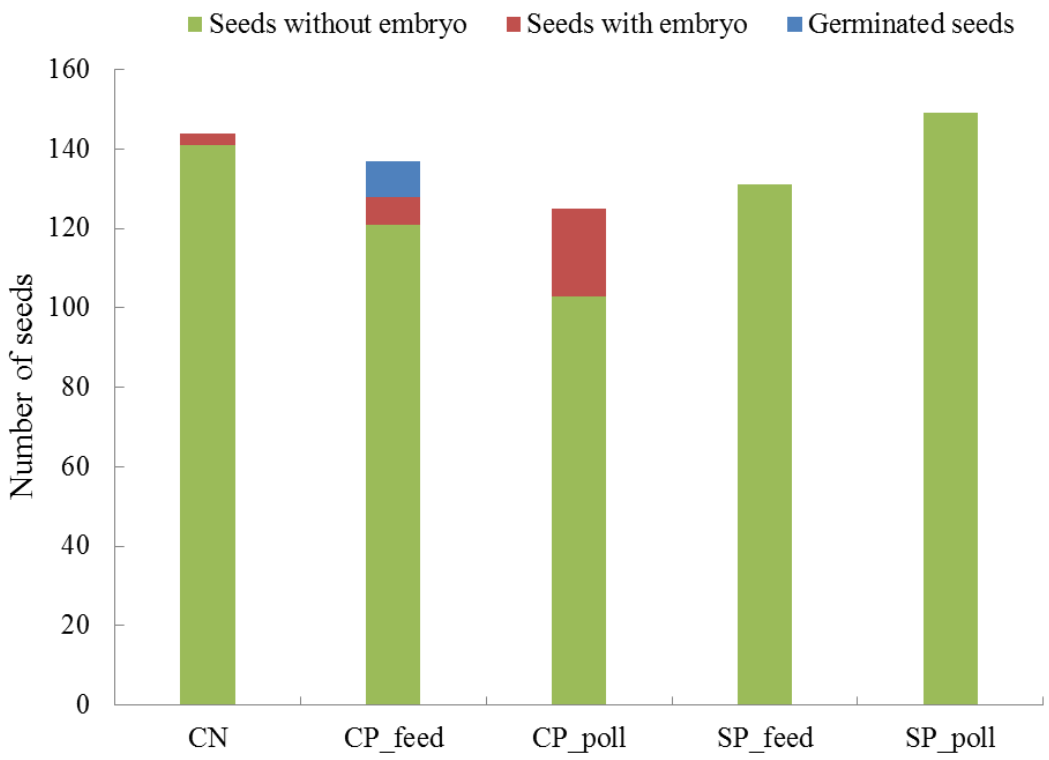

Fig.4. Germination test of *Trembleya laniflora* Cong. (Melastomataceae) from seeds of different treatments (CN = Control; CP_feed = cross-pollination with feed pollen; CP_poll = cross-pollination with pollination pollen; SP_feed = Self-pollination with feed pollen; SP_poll = Self-pollination with pollination pollen).

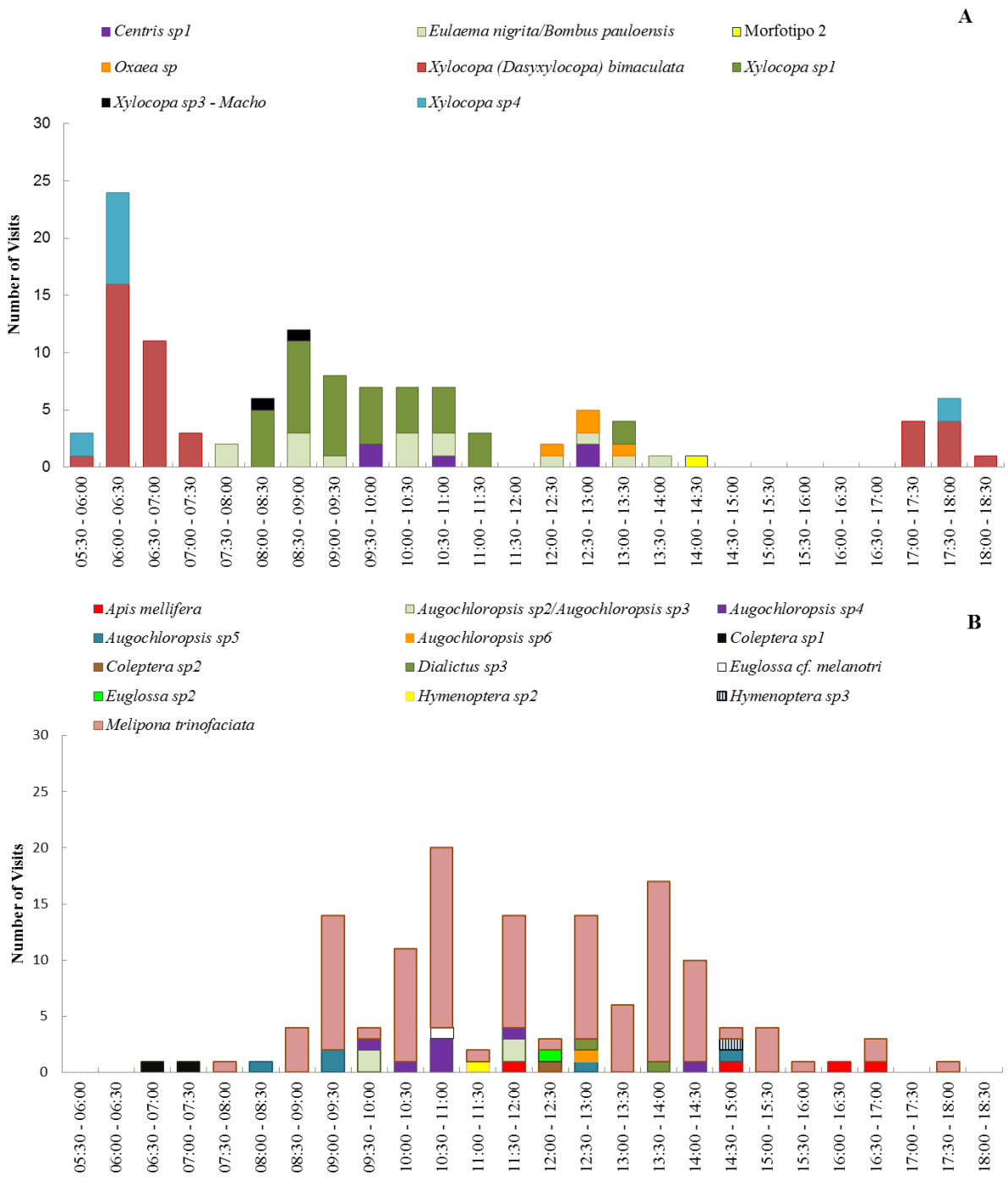

Fig.5. Frequency of visits by (A) pollinators (principal and secondary) and (B) non-pollinating visitors (flower visitor and pollen theft) to flowers of *Trembleya laniflora* Cong. (Melastomataceae) at Serra do Cipó (Minas Gerais, Brazil).

8. SUPPLEMENTARY INFORMATION

8.1. Tables

Table 1. Results of circular statistical analyses testing for the occurrence of seasonality in the reproductive phenology of *Trembleya laniflora* (Melastomataceae) in *campo rupestre* at Serra do Cipó (state of Minas Gerais, Brazil). Rayleigh test was performed for the significance of the mean angle or mean date.

	Floral bud		**Anthesis**		**Immature fruit**		**Mature fruit**	
	Onset	**Peak**	**Onset**	**Peak**	**Onset**	**Peak**	**Onset**	**Peak**
Mean Vector (μ)	154.172°	177.424°	169.044°	190.341°	187.607°	230.417°	260.747°	286.027°
Mean Date	05/Jun	28/Jun	17/Jun	12/Jul	09/Jul	21/Aug	21/Sep	17/Oct
Length of Mean Vector (r)	0.97	0.98	0.96	0.96	0.96	0.97	0.97	1.00
Circular Standard Deviation	13.15°	12.731°	15.69°	16.581°	15.761°	14.336°	13.21°	nc
Rayleigh Test (Z)	29.409**	25.699**	28.760**	25.751**	28.741**	22.544**	25.602**	13.000**

**** p< 0.0001**

Table 2. Floral visitors, pollinator (main and secondary) and visitor (flower visitors and pollen theft) species, of *Trembleya laniflora* Cong. (Melastomataceae) at Serra do Cipó (state of Minas Gerais, Brazil). Observations conducted in July−August 2014 and July 2015.

Floral Visitors	***Forage time***	***Classification***
Pollinator species		
Centris sp1	Diurnal	Secondary Pollinator
Centris sp2	Diurnal	Secondary Pollinator
Eulaema nigrita Lepeletier, 1841/*Bombus pauloensis* Friese, 1931	Diurnal	Secondary Pollinator
Morfotipo 1	Diurnal	Secondary Pollinator
Morfotipo 2	Diurnal	Secondary Pollinator
Oxaea sp	Diurnal	Secondary Pollinator
Ptiloglossa sp1 (Colletidae) - Fêmea	Crepuscular	Secondary Pollinator
Xylocopa (Dasyxylocopa) *bimaculata* Friese, 1903	Crepuscular	Main Pollinator
Xylocopa sp1	Diurnal	Main Pollinator
Xylocopa sp3 - Macho	Diurnal	Secondary Pollinator
Xylocopa sp4	Crepuscular	Main Pollinator
Xylocopa sp5	Diurnal	Secondary Pollinator
Visitor species		
Apis mellifera	Diurnal	Pollen theft
Augochloropsis sp1	Diurnal	Pollen theft
Augochloropsis sp2/*Augochloropsis* sp3	Diurnal	Pollen theft
Augochloropsis sp4	Diurnal	Pollen theft
Augochloropsis sp5	Diurnal	Pollen theft
Augochloropsis sp6	Diurnal	Pollen theft
Coleptera sp1	Diurnal	Flower Visitor
Coleptera sp2	Diurnal	Flower Visitor
Dialictus sp3	Diurnal	Pollen theft
Euglossa cf. *melanotri*	Diurnal	Pollen theft
Euglossa sp2	Diurnal	Pollen theft
Exomalopis sp1	Diurnal	Pollen theft
Hymenoptera sp2	Diurnal	Flower Visitor
Hymenoptera sp3	Diurnal	Flower Visitor
Melipona trinofaciata	Diurnal	Pollen theft

8.2. Figures

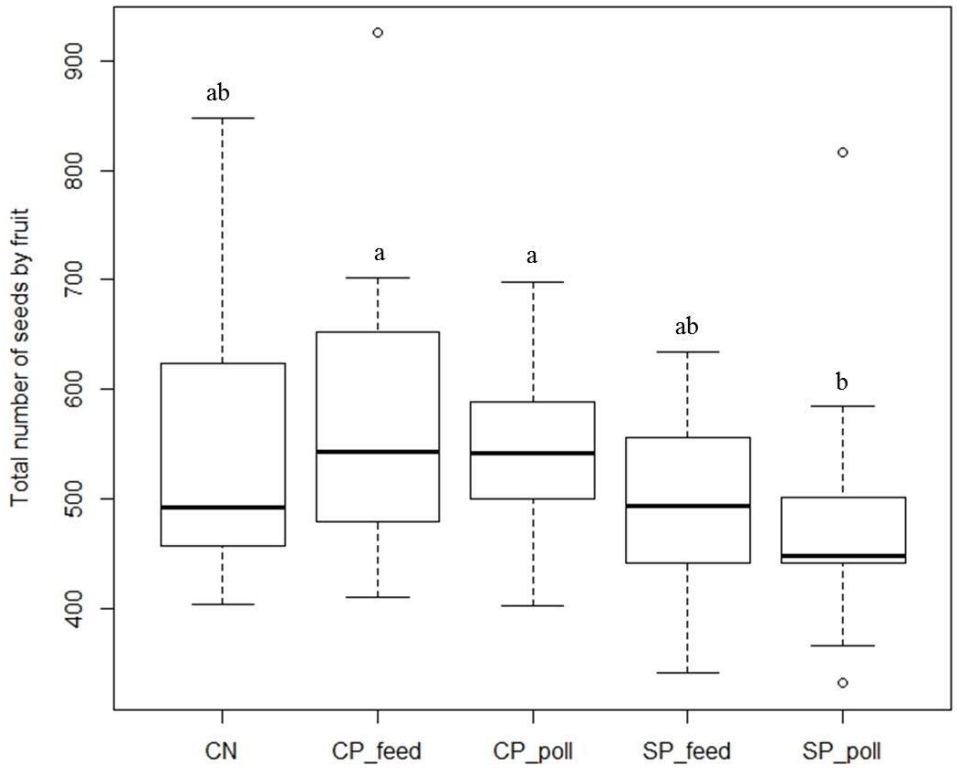

Fig.1. Boxplot for total number of seeds by fruit (N = 20 fruits) of *Trembleya laniflora* (Melastomataceae) resulting from different treatments of the reproductive system. Different letters indicate significant differences among treatments. CN = control; CP_feed = cross-pollination treatment with feed pollen; CP_poll = cross-pollination treatment with pollination pollen; SP_feed = self-pollination treatment with feed pollen; and SP_poll = self-pollination treatment with pollination pollen.

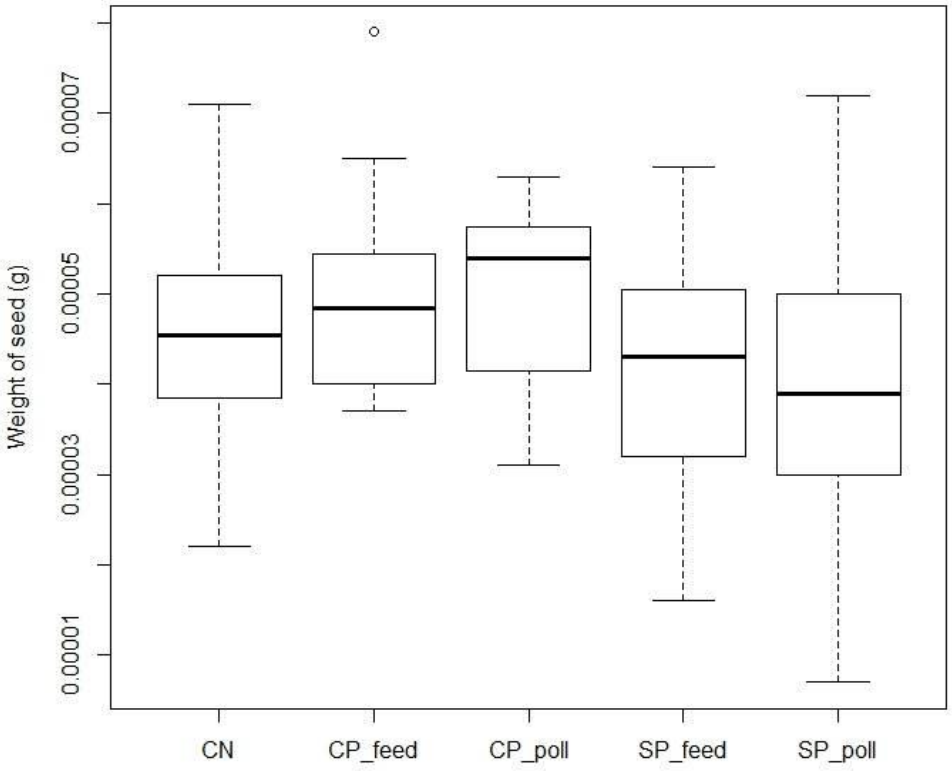

Fig.2. Boxplot for weight of seeds resulting from different treatments of the reproductive system of *Trembleya laniflora* (Melastomataceae). CN = control; CP_feed = cross-pollination treatment with feed pollen; CP_poll = cross-pollination treatment with pollination pollen; SP_feed = self-pollination treatment with feed pollen; and SP_poll = self-pollination treatment with pollination pollen.

Capítulo 2

Intraspecific variation and constraints on reproduction of *Trembleya laniflora* (Melastomataceae): an endemic species, naturally isolated on rocky outcrops

Chapter 2 – Intraspecific variation and constraints on reproduction of *Trembleya laniflora* (Melastomataceae): an endemic species, naturally isolated on rocky outcrops

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ABSTRACT

Plants are variable organisms and intraspecific differences in reproductive traits of species are important to their ecological interactions and evolution. *Trembleya laniflora* Cong. (Melastomataceae: Microlicieae) is a narrow-endemic, non-apomitic and self-incompatible species with distribution restricted to rock outcrops of the *campo rupestre*, an old growth mountain grassland from Southeastern Brazil. *Trembleya laniflora* interbreeding is dependent of a specialized buzz-pollination system mediated by crepuscular bees, and the floral traits are related with pollinators' foraging behavior. Here, we tested for the occurrence of intraspecific variations in the reproductive ecology (floral biology, phenology, and pollination) and reproductive success of three naturally isolated populations of *T. laniflora*. We aim to understand which factors (biotic and abiotic) explain the interpopulation differences. We randomly sampled individuals inside 1 hectare plots marked in three sites at distinct altitudes localized in the Serra do Cipó, South of Espinhaço Mountain range, Minas Gerais state. We tested for variations in reproductive phenology (at least 31 individuals/site), floral biology (at least 28 floral buds/site), and pollination ecology (all flowering individuals) through periodic observations from January to December 2014. The populations differed regarding all the reproductive aspects evaluated and their reproductive success. The main differences were found between the lowest site and the other two high elevation sites. Temperature was associated with the phenological time and the production of reproductive structures, while the biotics variables (numbers of flowers, frequency of the pollination events, and anthesis duration) explained the production of mature fruits and, consequently, the differences in the reproductive success. Our results suggested that temperature has an indirect relationship with the reproductive success of the populations by driving variations in the time of reproductive phenology, whereas, biotic interactions were the main selective factors of flowering opening time, determining the exhibition of flowers to main pollinators and pollen predators, direct affecting the reproductive success of each population. We conclude that abiotic and biotic factors act together as strong selective pressures in the reproductive biology of *Trembleya*, driving the intraspecific variations in the flowering and fruiting times, affecting the reproductive success of naturally isolated populations in space.

Keywords: Abiotic and biotic selective forces, crepuscular bees, reproductive phenology, rupestrian grassland.

1. INTRODUCTION

Plants are highly variable organisms and changes in key reproductive characters such as flowering and fruiting phenology (timing, duration, intensity and, synchrony) are ecologically relevant for both, plants and the animals they interact (Augspurger, 1981; Ollerton and Lack, 1992; Elzinga et al., 2007; Herrera, 1995a, 2009). Morphological (size, shape, numbers of structures), chemical (floral scent, nectar concentration, chemic and nutritional fruit composition), and ecological (diversity and abundance of pollinators, phenology timing) traits are plastic characters which vary among individuals of a species (Stebbins, 1950; Real and Rathcke, 1991; Herrera, 1988, 1995b, 2009; Otárola et al., 2013; Zywiec et al., 2012, Braunshmid et al., 2017). Around 25% of the variation in vegetative traits within communities and 32% of the total of trait variation occurring among plant communities are associated to intraspecific trait variations (Sierfert et al., 2015). Moreover, intraspecific variations on morphological, physiological and phenological traits may influence the plant fitness affecting the growth, reproduction, and survival of individuals, modifying ecological and evolutionary processes from population to community level (Bolnick et al., 2011; Violle et al., 2007, 2012; Sierfert et al., 2015).

Phenological patterns can differ among individuals (Herrera, 1988, 1992; Newstron et al., 1994; Sakai et al., 2005), and plant phenology is under the selective pressure of abiotic (temperature, photoperiod, precipitation, soil composition), biotic (e.g. competition for pollinators and dispersers), physiologic, and phylogenetic factors (Wright and Van Shaik, 1994; Morellato et al., 2000; Borchert, 2002; Sakai, 2001; Elzinga et al., 2007; Staggmeier et al., 2010; Brito et al., 2017). Environmental factors securely signalize the best climatic seasons and the more favorable conditions for the reproduction of plant species mediated by plant-animal interactions (Rathcke and Lacey, 1985; Wright and Van Shaik, 1994; Morellato et al., 2000; Elzinga et al., 2007). Furthermore, flowering time represents an adaptation of pollinator-dependent plants to periods of higher abundance of their biotic pollen vectors and, consequently, plant-pollination interactions play a strong selective pressure on plant's reproductive phenology (Waser, 1983; Rathcke and Lacey, 1985; Van Schaik et al., 1993; Brito et al., 2017).

The density of flowers and fruits in time and space has also a crucial role in the reproductive success of a species due to its importance to enhance attractiveness of plants to pollinators (Augspurger, 1981; Rathcke and Lacey, 1985; Maroho, 2002). Divergences in flowering time

can reduce intraspecific pollen transfer by biotic vectors acting as a pre-mating isolation mechanism of individuals within and among natural populations (Levin, 1971; Rathcke and Lacey, 1985). Furthermore, isolated individuals that present asynchronous flowering in relation to its population are often less visited by effective pollinators, reducing the reproductive success (Augspurger, 1981, 1983; Rathcke and Lacey, 1985; Fox and Kelly, 1993; Maroho, 2002; see also Ollerton and Lack, 1992).

The *Campo rupestre sensu stricto* is a species-rich, old-growth, vegetation mosaic dominated by grasslands associated to vegetations on rocky outcrops, which occurs predominantly above 900 m up to 2100 meters (above sea level) in quartzite, sandstone and ironstone bedrocks on the Espinhaço Mountain range and a few other locations (Alves & Kolbek, 2010; Silveira et al., 2016). The flora high diversity and endemism is associated to influence of the surrounding biomes (*Cerrado*, *Caatinga* and Atlantic Forest), the high soil diversity, the heterogeneity of habitats, and the isolation of vegetation on rocky outcrops across the mountain tops (Giulietti et al., 1997; Benites et al., 2007; Silveira et al., 2016). Melastomataceae is one of the species-richest families in the *campo rupestre*, represented mostly by endemic genus as *Cambessedesia*, *Chaetostoma* and, *Lavoisiera* (Giulietti et al., 1987; Alves and Kolbek, 2010). *Trembleya laniflora* Cong. (Microlicieae) is a non-apomictic, self-incompatible, endemic shrubby species restricted to the rock outcrops of the *campo rupestre* of south Espinhaço Mountain, Minas Gerais, Southeastern Brazil (Martins, 1997; Soares and Morellato, 2017). The large, showy and white-colored flowers of nocturnal anthesis are associated to a highly specialized buzz-pollination system mediated by large bees with crepuscular foraging behavior (Soares and Morellato, 2017).

Considering the endemism and natural isolation of *T. laniflora* on rock outcrops of *campos rupestres* (Martins, 1997), the direct relationship among the species floral traits and the dependence of interbreeding mediated by specialized biotic vectors of pollination (Soares and Morellato, 2017) and, the importance of intraspecific trait variation to ecology and evolution of plant species and communities (Violle et al., 2012; Sierfert et al., 2015), here we aimed to evaluate the occurrence of intraspecific variations in the reproductive ecology (floral biology, phenology, and pollination) of three naturally isolated populations of *T. laniflora*. We tested for the possible drivers of changes and its relevance to the reproduction of these narrow-endemic species. We addressed three specific questions (i) Do the populations of *Trembleya laniflora* vary in their phenology (time, duration, synchrony), flower biology (anthesis timing,

flower lifespan), and pollination (frequency of visits by pollinators, composition of pollinators, and floral visitors)? (ii) Do the differences relate to the populations' reproductive success? and, (iii) What factors – abiotic or biotic - drive the variations on the reproductive success of these naturally isolated plant populations on rock outcrops?

2. MATERIALS AND METHODS

2.1. Study area

We studied from January to December 2014 three populations of *Trembleya laniflora* situated in the *campo rupestre* within the environmental protection area of Morro da Pedreira, Serra do Cipó (SC), South of the Espinhaço Mountain Range, state of Minas Gerais, southeastern Brazil. The three study sites are locally referred as Cedro (CE): the low-altitude site at 1101m, coordinates 43º34'56"W; 19º14'16"S; Pedra do Elefante (PE): mid-altitude site at 1255m and 43º33'20"W; 19º17'34"S, and the Quadrante 16 (Q16): the high-altitude site at 1303 m (43º35'31"W; 19º17'42"S). All sites are *campo rupestre sensu stricto* (Silveira et al., 2016) vegetation mosaics dominated by a grasslands matrix and associated rocky outcrops, differing mainly in relation to the altitude (Rocha et al., 2016) (Figures 1 and 2). According to Köppen (1931), the climate at the Serra do Cipó region is mesothermal (Cwp) with well-defined dry and wet seasons, grouped in four main periods: dry season (May to September); post-dry season (October); rainy season (November to January); and post-rainy season (February to April) (Madeira and Fernandes, 1999; Silveira et al., 2016).

2.2. Local climate

Environmental data of photosynthetic active radiation (PAR, W/m^2), precipitation (mm), temperature (ºC) and, relative humid (RH, %) were obtained from climate stations (HOBO U30 Station GSM-TCP Model) localized at each study site (CE, PE, and Q16); for details see Fernandes et al. (2016). To provide daily and monthly weather values, data were aggregated using the function *ddply* of *plyr* package available in R (R Development Core Team, 2011).

2.3. Study species

Trembleya laniflora Cong. (Melastomataceae - Microlicieae) is an endemic shrub to treelet species of occurrence restricted to the rock outcrops of the *campos rupestres* South of Espinhaço Mountain range (Martins, 1997). *Trembleya laniflora* distinct floral traits (large, showy and, white-colored flowers of nocturnal anthesis) are associated to the specialized

buzz-pollination system mediated by large crepuscular bees (Soares and Morellato, 2017), and the dry, capsular, dehiscent fruits produce many diminute self-dispersed seeds (Martins, 1997; Soares and Morellato, 2017). The reproduction occurs during the dry, post-dry and beginning of the rainy seasons, with flowering mainly from May to October and fruiting from June to December (Soares and Morellato, 2017).

2.4. Reproductive Biology Measurements

2.4.1. Phenology – For the phenology monitoring, at each study site, we randomly sampled at least 31 reproductive adults of *T. laniflora* inside 100 m x 100 m (1 ha) plots (Table S1, Supplementary information) monitored at weekly to monthly intervals from January to December 2014. We conducted biweekly phenological observations during the main flowering period (July and August). We quantified the phenology of each populations, by counting the number of reproductive structures (floral buds, flowers, immature fruits and mature fruits) on five marked branches of the selected *T. laniflora* individuals and, later estimate to all crown (Ollerton and Dafni et al., 2005). Moreover, the onset and peak dates of each populations' phenophases were represented, respectively, by the observed mean date of first emergence and of peak production (counting) of floral buds, flowers, immature and mature fruits by all monitored individuals of each population (see Morellato et al., 2000). Due to the fires in October of 2014 and the consequent death of 15 of the 31 sampled individuals at high-altitude site Q16 and of 14 of the 32 sampled individuals at mid-altitude PE, the phenological observations from September to December 2014 refer to 16 and 18 individuals, respectively (Table S1, supplementary information).

We defined the following reproductive parameters: (i) the *monthly total production* as the sum of reproductive structures produced per month (or fortnightly) by individuals, partitioned by number of individuals of the population in a given phenophase; (ii) *monthly individual production* as number of structures counted per month in each individual of a given population; (iii) *anual production* as sum of montly individual productions, iv) *individual production* as the sum of reproductive structures produced by individual all year round; (v) *maximal production* as the maximum amount of floral bud, flower and fruits (immature and mature) counted by individual; (vi) *damaged structures* as the maximum amount of damaged floral buds and flowers counted for each individual of population, (vii) *reproductive success* as the ratio of the sum of mature fruits by the sum of flowers counted on each sampled individual.

2.4.2. Floral biology – To evaluate the occurrence of intraspecific variation in the floral biology of *T. laniflora* we observed the development of flower buds previously tagged in three individuals randomly chosen inside each study site plot. During five consecutive days, we supervised in the CE, PE, and Q16 populations, respectively, 42, 28, and 40 marked flower buds. The surveys were made from dawn to dusk (05.30–18.30 h) through senses carried out each hour, except during the dawn, when the observations occurred every 30 minutes. Due to the floral longevity of three days (mainly), on the fourth and fifth day of observations we made only four surveys throughout the day (06.00 h; 07.00 h; 12.00 h and 18.20 h) (see Soares and Morellato, 2017 for more details).

2.4.3. Pollination ecology – During July and August 2014 we registered the interactions among individuals of *T. laniflora* in the three sampled populations and its flower visitors (240 hours of observation). We monitored the flower visitors of selected flowering individuals sampled for phenological observations (see Table S1, supplementary information). For this purpose, we randomly carried out 10-minute surveys of each blooming individual between 05.30 h and 18.30 h (dawn to dusk) on each day of observation (Herrera, 1995b). In these surveys, we quantified the available resources (number of open flowers) and, the number and identity of flower visitors (pollinators and other visitor species).

We first classified the visits as legitimate or visits by effective pollinators, and illegitimate or flower visits not resulting in pollination (Inouye, 1980). Effective pollinators were classified as main or secondary according to their collection and foraging behavior, and their frequency of visit to *T. laniflora* flowers (Inouye et al., 1994; Rosas-Guerrero et al., 2014; Dafni et al., 2005; Soares and Morellato, 2017). We considered as main pollinators the large bee species ($\geq$ 2.5 cm) that were capable of vibrating both types of anthers (feeding and pollination anthers) concurrently contacting the stigma (Soares and Morellato, 2017), and that contributed to at least ten percent ($N \geq 10\%$) of the total of visits. Large bees that possessed vibration capability and contacted the reproductive parts less than 10 percent ($N < 10\%$) of the total visits were considered as secondary pollinators. Small bees ($\leq$1.5 cm) that were not capable of vibrating both types of anthers concurrently, pollen thefts, pollen robbers, and other insects that visited the flowers but did not contact the stigma were considered illegitimate flower visitors (Inouye, 1980; Soares and Morellato, 2017).

2.5. Data analyses

To test for differences on the local climate (PAR, precipitation, temperature and relative humidity) of the three study sites we applied Generalized Linear Models (GLM) using the sites as the predictor variable and the dayly weather values as response variable (Nelder and Wedderburn, 1972). We assumed Gaussian distribuition for GLM errors. To test for correlations among the monthly local climate (PAR, precipitation, temperature and relative humidity), and among the monthly local climate and the reproductive phenology (monthly individual production) we applied Spearman's correlation test using the *stats* function (R Development Core Team, 2011). Due to the strong autocorrelation ($\rho > 0.34$) found among the monthly values of environmental variables and the higher correlation of local temperature with the monthly individual production of flower buds, flowers and fruits of *T. laniflora*, we defined the temperature as the predictor abiotic variable on all posterior analyses (Table S2, supplementary information).

We tested for seasonal patterns and compared the flowering and fruiting phenology (onset and peak dates) of populations from the three study sites using circular statistical analysis following Morellato et al. (2000, 2010). We tested for seasonality by testing the significance of the mean angle or mean onset and peak dates of each phenophase by applying the Rayleigh (z) test. A significant mean angle or date indicates that the distribution of peak or onset dates is not uniformed among the year and the reproductive phenological pattern is seasonal (Morellato et al., 2000); the two-sample Watson-Williams tests (F) were further applied to compare whether the seasonal patterns differed among populations. The length of the resulting mean vector (r) indicates how grouped the dates are around the mean angle and indicates the degree of phenological seasonality or synchrony of the phenophases for each population (Morellato et al., 2000). The mean vector r ranges from 0 (no seasonal or asynchronic) to 1 (highly seasonal or synchronic). All analyses were performed in Oriana 4 (Kovach, 2011).

Differences on (i) reproductive success, (ii) damaged structures, (iii) legitimate visits (number of plant-pollinator interactions) and, (iv) illegitimate visits (number of plant-visitor interactions) of the populations were tested utilizing GLMs (Gaussian errors distribution). The populations (sites) were used as the predictor variable in the models (Nelder and Wedderburn, 1972). Interpopulation differences (among sites) on the: (i) monthly total production, (ii) individual production and, (iii) maximal production of floral buds, flowers and fruits (immature and mature) were tested through Kruskal-Wallis test. In addition, variation on the

number of floral antheses (flowers open) in each observed period (hour) were tested with a two-sample proportion test and variations in the flowers' lifespan through ANOVA and Tukey Test (Zar 1996).

To evaluate if biotic and abiotic factors can explain the differences in the reproduction of *T. laniflora* populations we generated GLMs using Poisson distribution to the errors (Nelder and Wedderburn, 1972). Firstly, we tested for possible effects of temperature (abiotic variable) in the reproductive phenology utilizing the monthly individual production of floral bud, flower, immature and, mature fruit, as response variable and, the temperature on the observation day, as the predictor variable on the models. Subsequently, we evaluated what factors influence the mature fruit production and, consequently, the reproductive success of each population. We utilized the sum of the mature fruits produced by each individual as the response variable, and (i) the temperature over the flowering peak (temperature on peak date of individual), (ii) individual anthesis duration (number of days with flower), (iii) individual flowers production and, (iv) the frequency of flower visits by pollinators (proportion of legitimate visits by number of surveys in each plant), as predictor variables on GLM. All analyses were realized using the R statistical software (R Development Core Team, 2011).

3. RESULTS

3.1. Local climate

The study sites significantly differed to all the environmental variables, except precipitation (Figure S1 supplementary information). The high-altitude site (Q16) presented significant lower temperatures ($t = -5.46$, $p<0.001$) and higher PAR ($t=2.89$, $p=0.003$) and relative humidity ($t=2.91$, $p=0.003$), contrasting with the low-altitude (CE) and mid-altitude (PE) sites (GLMs, $p < 0.001$) (Figure S1, supplementary information).

3.2. Reproductive biology

3.2.1 Phenology

The three *Trembleya laniflora* populations presented significant, high seasonal patterns of flowering and fruiting ($r \geq 0.9$), concentrated during the dry and post-dry seasons, differing however regarding their mean dates (onset and peak) of phenological activity (Figures 3, 4 and 5; Table S1, supplementary information). We observed a significant delay of fifteen days

on the mean date of flower bud peak of the low-altitude site CE (July 20th), compared to the on the mean date of flower bud peak of the low-altitude site CE (July 20th), compared to the high-altitude, Q16 (July 5th) and, mid-altitude PE (July 13th) one (Figure 4D-F). In fact, the flower onset was about ten days earlier the for high- and mid-altitude populations at Q16 (June 30th) and PE population (June 27th), respectively (Figure 4G-I). We also observed a significant early peak flowering mean dates, above twelve days, at high- and mid-altitude populations (July 21th and 24th, respectively), while low-altitude mean peak flowering activity was August 6th (Figure 4J-L; Table S1, supplementary information). Likewise, we observed significant differences in the mean fruiting dates, about 15 days earlier at high- and mid-altitude populations than at low-altitude CE ones (Figure 5; Table S1, supplementary information). Populations from high- and mid-altitude sites were overall more similar, differing significantly only for the mean dates of mature fruit peak production (Figure 5J-L; Table S1, supplementary information).

The monthly total, individual and the maximum production of flower buds, flowers, and fruits (immature and mature) did not significantly differ between the three populations of *T. laniflora* (Table S3, supplementary information). However, individuals of mid-altitude PE presented a higher annual production of reproductive structures (140,547 flower buds, 141,079 flowers, 396,284 immature fruit and, 323,228 mature fruits) than low-altitude CE (125,015 flower buds, 117,596 flowers, 103,506 immature fruit and, 474 mature fruits) and high-altitude Q16 plants (98,391 flower buds, 105,005 flowers, 262,669 immature fruits and, 23,686 mature fruits). Mid-altitude PE individuals also remained in the flowering peak longer (two months) than those on the other two sites (Figure 3).

We found significantly higher average values of reproductive success for the populations observed at mid-altitude PE (0.37) and high-altitude Q16 (0.26) sites, than at low-altitude CE (0.15) ($t=4.36$, $p<0.001$, $R^2 = 0.18$) (Figure 6A). A significantly higher number (96,336) of damaged buds and flowers (predation) were observed at mid-altitude PE individuals than at Q16 individuals (30,728) and CE (2,196) ($t=2.81$, $p =0.006$, $R^2 = 0.08$) (Figure 6B).

3.2.2. Floral Biology

We found significant differences in the number of flowers opened during the main period of anthesis (18:30h-06:30h) among the three *T. laniflora* populations (Figure 7). Most of the 42 tagged flower buds at low-altitude CE individuals, opened during the night (31 or 73.8 %),

while for at mid- PE and high-altitude Q16 individuals, presented a similar proportion of nocturnal and sunrise anthesis (Figure 7). We also observed significant differences in the flower lifespans. Flowers of mid-altitude PE individuals remained open for a longer time (mean of 4 days) than flowers of the others two populations (mean of 3 days) ($F=88.8$, $p <0.001$; Tukey test, $p<0.001$) (Table S4, supplementary information).

3.2.3. Pollination Ecology

We observed 117 pollination events in high-altitude Q16 individuals, 47 in mid-altitude PE individuals, and 29 in low-altitude CE individuals. To all the populations, these events occurred mainly during the dawn (from 05:30h to 07:00h) and the dusk (from 17:30h to18:30h), and were performed by bee species with crepuscular foraging behavior (Figure 8). During these periods of dim-light, we observed 42% (49/117) of the visits by pollinators at high-altitude Q16, 60% (28/47) at mid-altitude PE, and 76% (22/29) at CE individuals. The number of legitimate visits by pollinator species at high-altitude Q16 individuals was significantly higher than observed on individuals at mid- PE and low-altitude CE ($z = 6.06$, $p < 0.001$, $R^2 = 0.14$) (Figure 6C).

The crepuscular bees, *Ptiloglossa* sp1 and *Xylocopa truxali*, were the most effective and main pollinators species at low-altitude CE, contributing together with 62% (N = 18) of the pollination events, followed by *Xylocopa* sp1 and *Xylocopa* sp2 (10.34% each). Three species *Eulaena nigrita/Bombus pauloensis* (10.34%), *Centris* sp2 (3.45%) and *Xylocopa bimaculata* (3.45%) were classified as secondary pollinators at CE (Figure 8A). In contrast, at mid- PE and high-altitude Q16 individuals *X. bimaculata* was the most effective and main pollinator species, contributing alone with 32% (N = 15) of observed events at PE and with 34% (N = 40) at the Q16 population (Figure 8B and 8C). At mid-altitude PE, besides *X. bimaculata*, *Centris* sp1 (21.28%) and *Ptiloglossa* sp1 (17.02%) were also classified as main pollinators, whereas *Xylocopa* sp1 (8.51%), *Xylocopa* sp4 (8.51%), *Centris* sp 2 (6.38%), *Eulaena nigrita/Bombus pauloensis* (4.26%) and *X. truxali* (2.13%) were raked as secondary pollinators (Figure 8B). At high-altitude Q16, the main pollinators were *X. bimaculata*, *Xylocopa* sp1 (32.48%) and *Xylocopa* sp4 (10.26%), the secondary pollinators were *Eulaena nigrita/Bombus pauloensis* (12.82%), *Centris* sp1 (4.27%), *Oxaea* sp (3.42%), *Xylocopa* sp3 (1.71%), and Morfotipo 2 (0.85%) (Figure 8C) (see Soares and Morellato, 2017 for more results).

Trembleya laniflora populations also differed significantly regarding the number of illegitimate visits by diurnal visitors, floral visitors, pollen robber, and pollen thief (t = 6.42, *p* <0.001, Figure 6D). The pollen robber *Trigona spinipes* contributed respectively with, 85.6% (N=268) and 75.2% (N= 91) of the total flower visits observed at CE and PE population. At the high-altitude Q16 population the pollen theft *Melipona trinofaciata* was the main floral visitor with 77.5 % of visits (107) (Table S5, supplementary information).

3.3. Abiotic and biotic effects

The temperature was significantly and negatively related to the amount of flower buds, flowers, and fruits (immature and mature) produced by *T. laniflora* individuals, and explained 39% of individual production of flower buds (GLM; z = - 611.7, $p<0.001$, $R^2 = 0.39$), 34% of flower production (GLM, z = - 609.7, $p<0.001$, $R^2 =0.34$), but just 11% of immature and mature fruits availability (GLM, z = - 555.9, $p<0.001$, $R^2 = 0.11$). However, we did not find a significant relationship between the mean temperature over the flowering peak period and production of mature fruits by *T. laniflora* individuals. Individual production of mature fruits was only significant and positively explained by biotic variables: individual anthesis duration (z=8.57, p< 0.001), individual production of flowers (z = 173.1, p< 0.001) and, frequency of visit by pollinators (z = 22.9, p<0.001) explained 44% of the individual production and, consequent, the reproductive success of *Trembleya* individuals ($p<0.001$, $R^2_{GLM} = 0.44$).

4. DISCUSSION

Our results showed that there are intraspecific variations on the production of reproductive structures (flower buds, flowers, and fruits), timing and duration of phenology and, in the frequency of flower visits by pollinators and also frequency and type of illegitimate visits. The different phenological responses of flowering and fruiting of *T. laniflora* isolated populations were driven by differences on the study sites` local temperatures during the reproductive season, with early flowering on high altitude sites. Moreover, the high dependence of *T. laniflora* on its effective pollinators, affected the production of mature fruits, and the highest reproductive success was related to a higher activity of flowering (duration and production) and frequency of legitimate flower visitors. Therefore, our findings support the idea of that abiotic factors, here the temperature, and biotic factors, effective pollinators, act in conjunction, as strong selective pressure shaping the reproductive biology

and driving the flowering and fruiting time of plants (Rathcke and Lacey, 1985; O'Neil, 1999; Elzinga et al., 2007; but see also Ollerton and Lack, 1992).

The highly seasonal and synchronous flowering of *T. laniflora* populations in the dry season was associated with their isolated distribution on rock outcrops, self-incompatibility, and the dependence of specialized buzz-pollination by crepuscular bees. Highly seasonal flowering patterns are described for pollinator-dependent Melastomataceae species from *campo rupestre* vegetation (Brito et al., 2017). In addition, synchronic phenological responses in time and space are known to increase plant reproductive success due to increase attractiveness of effective pollinators, a premise confirmed by our observation (Augspurger, 1980; 1981; Bawa, 1983; Rathcke and Lacey, 1985; Mahoro 2002). We therefore suggest that the high seasonality and concentration of flower production ensures a conspicuous floral display of *Trembleya laniflora* populations during a defined short time span, attracting more pollinators, reducing the expected negative effects of natural isolation on the reproductive success (Faegri and van der Pilj 1979; Augspurger, 1981; 1983; Van Schaik et al., 1993; Athayde and Morellato, 2014, Soares & Morellato, 2017).

In addition, on the late mean dates of onset and peak flowering and fruiting presented by low-altitude CE individuals compared to the others two mid- and high-altitude populations (PE and Q16) were associated to the local environmental conditions, mainly of temperature. Abiotic factors direct and indirectly affect the phenology and reproduction of plant species (Rathcke and Lacey, 1985; Van Schaik et al., 1993; Donnelly et al., 2011; Morellato et al., 2016; Mendoza et al., 2017). *T. laniflora* reproduce annually during the coolest period of the year maintaining, therefore, a strong relationship between reproductive phenology and the low temperatures during dry and post-dry-seasons (Soares and Morellato, 2017). Therefore, the differences observed on *T. laniflora* populations flowering dates have likely been drive by the differences of local temperatures among studied sites. Variations in microenvironmental conditions, specifically temperature and light, are described to influence reproductive phenology at *cerrado* and *campo rupestre* plant species (Aguilar et al., 2006; Burgess et al., 2006; Camargo et al,. 2011; Le Stradic 2012; Athayde and Morellato, 2013; Vogado et al., 2016).

The production of floral buds and flowers were strong and negatively related to temperature, indicating the high reproductive success observed in the mid- and high-altitude populations (PE and Q16) were associated to the lower temperatures in those sites. The low temperatures

stimulated the early flowering, anticipating the offer of numerous flowers to the pollinators during the most favorable conditions to pollen transfer by their specialized biotic vectors (Waser, 1983; Rathcke, 1983; Fenner, 1998; Elzinga et al., 2007). Therefore, local variations of temperature were driving interpopulation differences on flowering time (onset and peak date) and productivity, as well as on fruit set, affecting the reproductive success of *T. laniflora* populations.

Mature fruit production and consequent seed set was explained mainly by flowering phenology (higher individual production of flowers and longer anthesis duration) and pollination interactions (frequency of visits). Even considering the numerous damaged structures, the highest reproductive success was observed in mid-altitude PE individuals. PE population presented the greater amount of floral buds and flowers, longer flower lifetime and, longer flower peak duration. The plants most frequently visited by pollinators (high-altitude Q16 population) also presented high reproductive success. Therefore, the reproductive success on *T. laniflora* populations was strongly influenced by flowering activity and plant-pollinator interactions. We suggested that in addition to the effects of temperature on timing and productivity of the *Trembleya* individuals, differences on reproductive output and success of their populations are directly related to the exhibition very attractive flowering display, flower density, and longer duration of the flowers, enhancing the plant attractiveness to their pollinators and, satiating the robbers and thieves of floral rewards (Faegri and van der Pilj, 1979; Waser, 1983; Rathcke, 1983; Rathcke and Lacey, 1985; van Schaik et al., 1993; Fenner, 1998). The expected disadvantaged due to high frequency of visits by pollen robber species (antagonist interactions) observed for low-altitude individuals of *Trembleya*, were therefore minimized by the increased floral display and attraction of the crepuscular effective pollinators (Rathcke, 1983; Hopkins et al., 2000; Somanathan and Borges, 2001; Elzinga et al., 2007).

In recent research, Soares and Morellato (2017) suggest the highly specialized buzz-pollination system of *T. laniflora* is related to the timing of flower opening associated with of crepuscular foraging behavior of bees. Additionally, due to the high number of embryoless and non-viable seeds and, the low germinability described to *T. laniflora* (Rodrigues and Silveira, 2013, Soares and Morellato, 2017) and Melastomataceae species of *campo rupestre* (Dayrell et al., 2017), we suggest that the intraspecific variations observed on floral biology and phenology are most likely associated with distinct strategies to attract crepuscular bees,

reduce loss of floral rewards by predation and, enhance pollination effectiveness (Rathcke 1983; Somanathan and Borges, 2001). Although several groups of bees developed nocturnal and crepuscular foraging behavior there is yet little evidence of their efficiency as pollinators and, the occurrence of specific adaptations of plants to pollination mediated by crepuscular bee species is also unclear (Hopkins et al., 2000; Somanathan and Borges, 2001; Wcislo et al., 2004; Franco and Gimenes, 2011; Cordeiro et al., 2017; Soares and Morellato, 2017).

In conclusion, we demonstrated that populations of *T. laniflora* under distinct microenvironmental conditions differed in their responses to abiotic and biotic selective factors which directly or indirectly reflected on reproductive success. Interactions with pollinators were the main selective pressure on flowering time of *Trembleya* populations driving the exhibition of flowers (synchrony, duration, production, timing floral opening, and flower lifespan) to the main pollinators and anthers predators, and having direct effects on the reproductive success (Augspurger, 1981; Rathcke and Lacey, 1985; Somanathan and Borges, 2001; Maroho, 2002). Abiotic factors, mainly temperature, had an indirect relationship with the reproductive success of the populations by driving the variations on onset and peak dates of flowering and fruiting along the altitudinal range. Moreover, intraspecific differences in the reproductive traits of *T. laniflora* populations were associated with species adaptations to a highly specialized buzz-pollination system mediated by crepuscular bees, and to its endemism, and natural isolation on rocks outcrops. Our findings indicated that abiotic and biotic factors act together as strong selective pressures of reproductive biology of *Trembleya*, driving the intraspecific variations in the flowering and fruiting times, affecting the reproductive success of populations naturally isolated in space.

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7. FIGURES

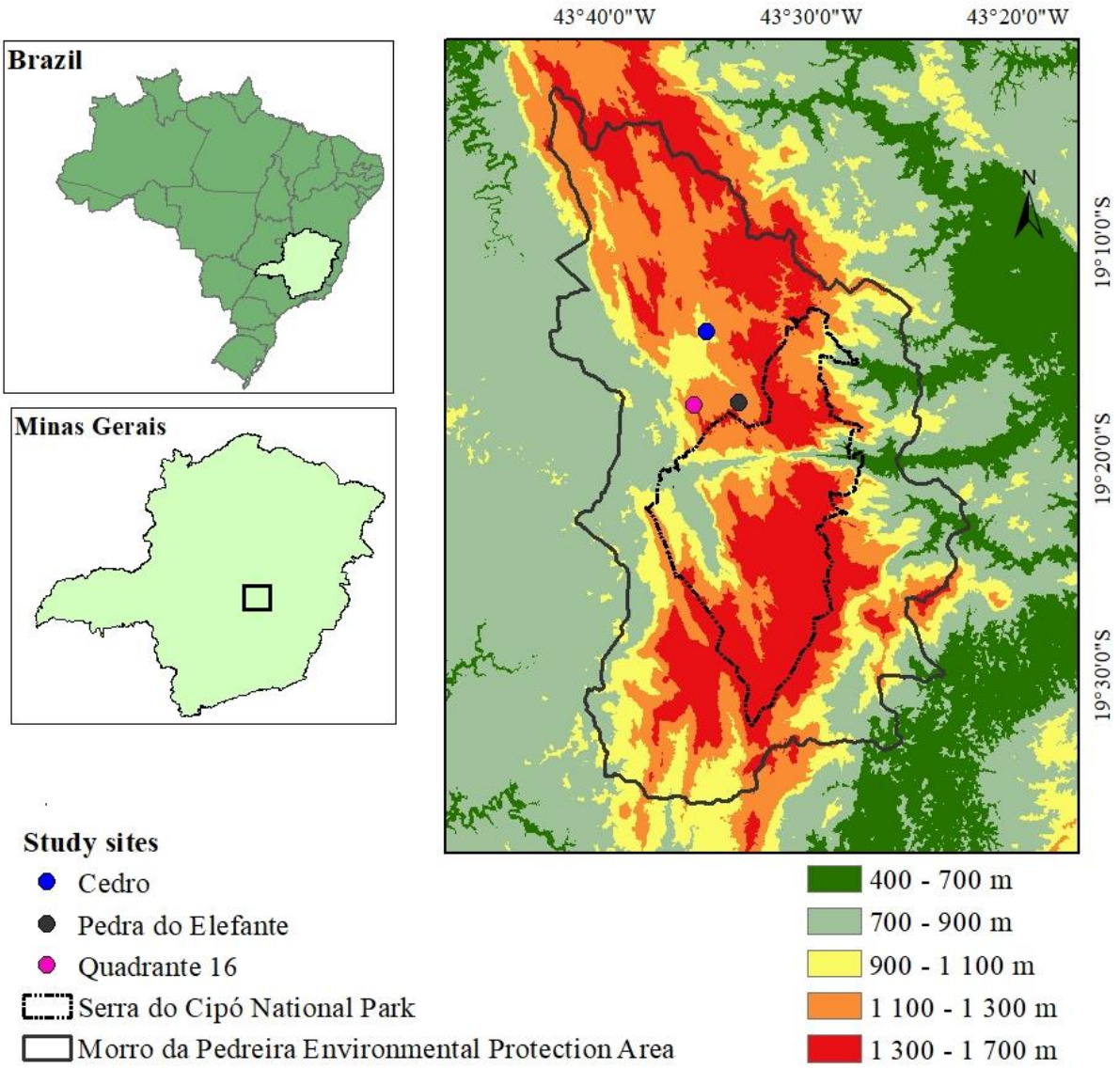

Fig.1. Localization of the studied sites low-altitude Cedro (1101 m), mid-altitude Pedra do Elefante (1255m) and, high-altitude Quadrante 16 (1303m) inside of the Morro da Pedreira Environmental Protection Area, Serra do Cipó, Minas Gerais state, southeastern Brazil. Fonte: adapted from Alvarado et al., 2017.

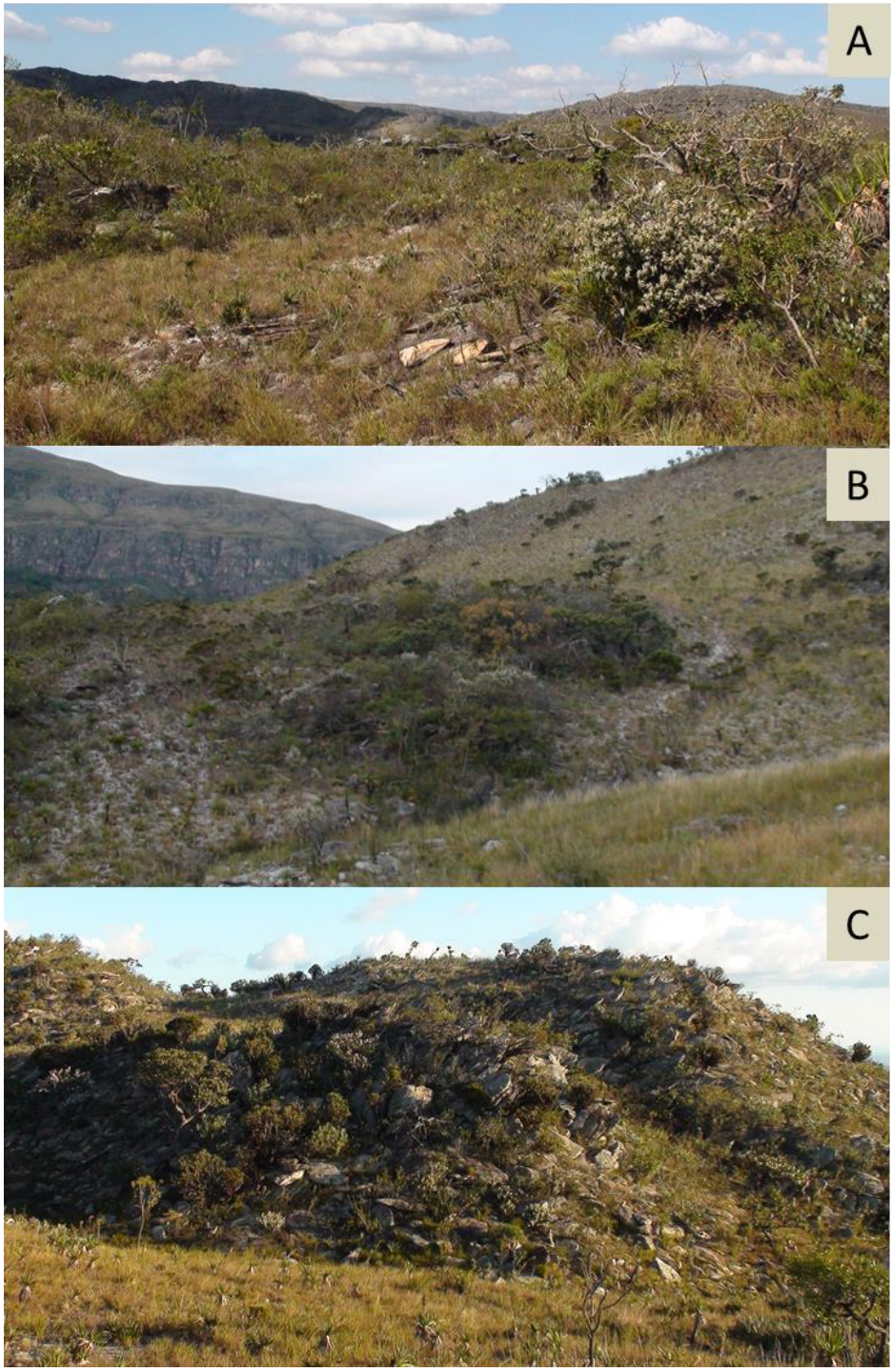

Fig.2. *Campo rupestre* sensu stricto study sites **(A)** low-altitude Cedro (1101m), **(B)** mid-altitude Pedra do Elefante (1255m) and **(C)**, high-altitude Quadrante 16 (1303 m) in Serra do Cipó, southeastern Brazil. Photos by Natalia C. Soares.

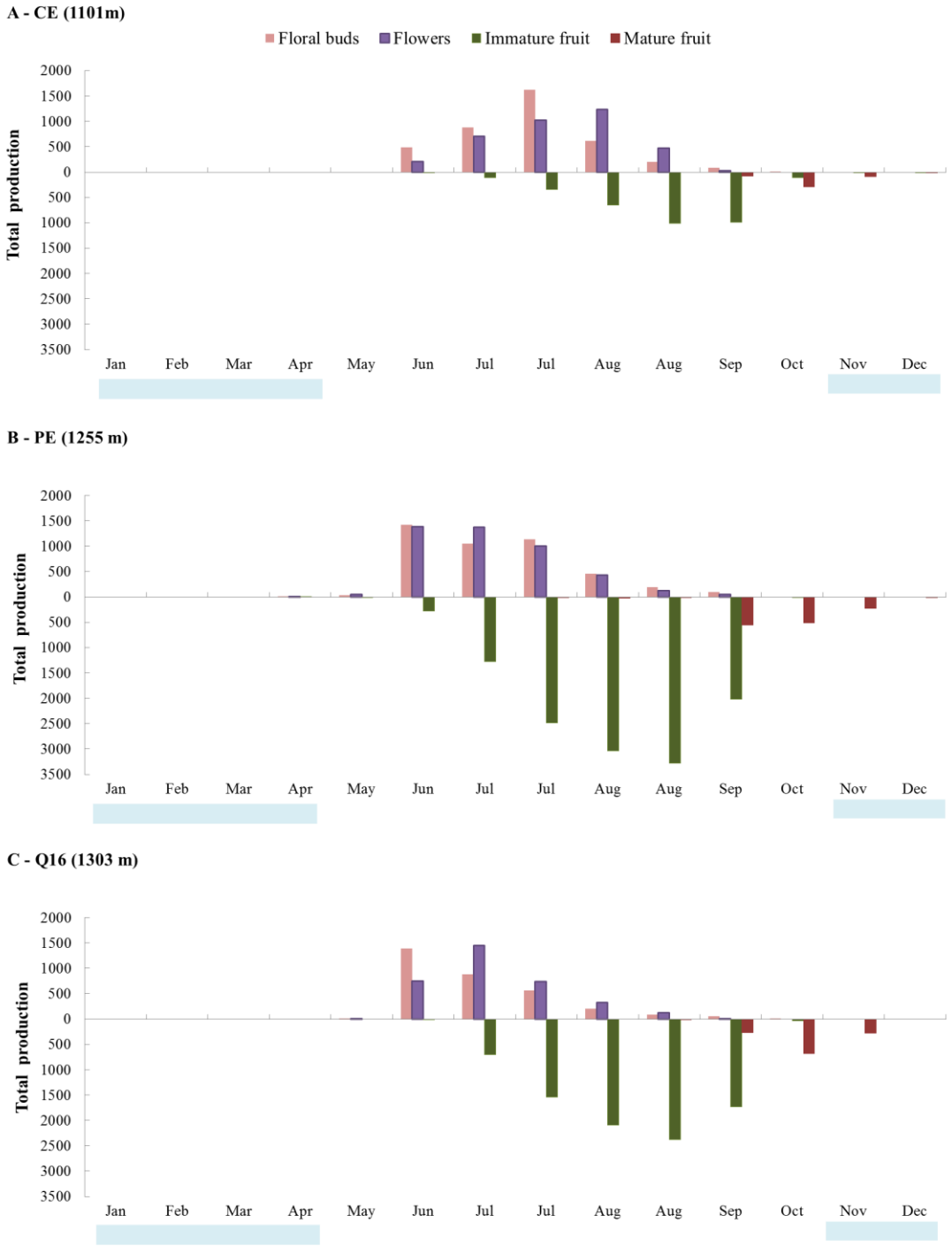

Fig.3. Monthly total production of reproductive structures (flower buds, flowers, immature and mature fruits) of *Trembleya laniflora* Cogn. (Melastomataceae) in three *campos rupestres* sites at Serra do Cipó, southeastern Brazil. **(A)** low-altitude site - Cedro (CE), **(B)** = mid-altitude site - Pedra do Elefante (PE), and **(C)**, high altitude site - Quadrante 16 (Q16). Blue bars indicate the wet season.

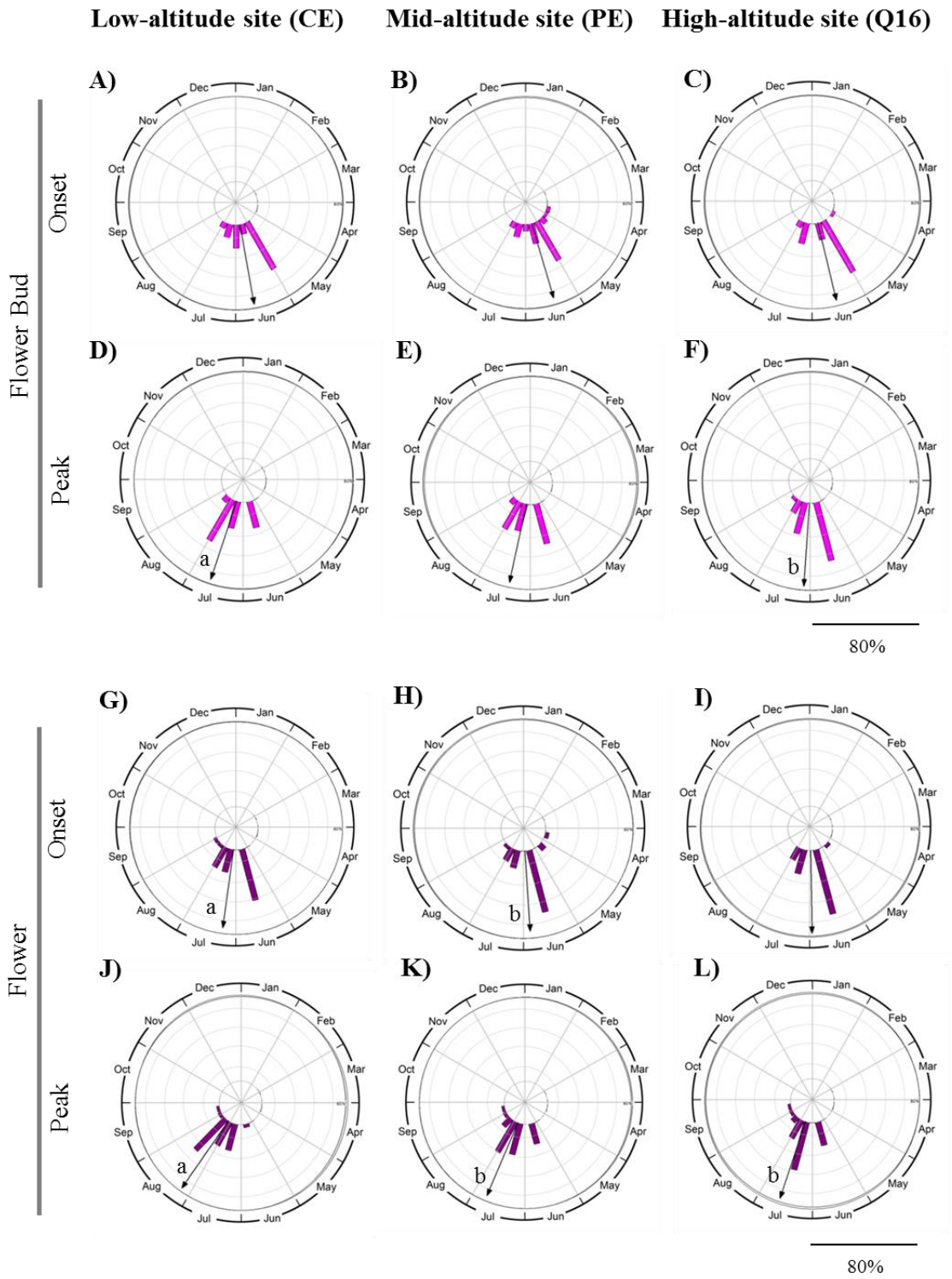

Fig.4. Circular distribuition of onset and peak dates for flower buds **(A-F)** and flowers **(G-L)** based on the mean pattern from January to December 2014 for *Trembleya laniflora* individuals sampled in the three *campos rupestres* sites, low- (CE), mid- (PE), and high-altitude site (Q16), at Serra do Cipó, southeastern Brazil. All mean angles are significant (Rayleigh test, $p < 0.001$) and are represented by the arrows. The length of the mean vector r (0 to 1) indicates how grouped the dates are around the mean angles. Different letters (a, b) indicate of significant differences (Watson-Willians test, $p < 0.05$).

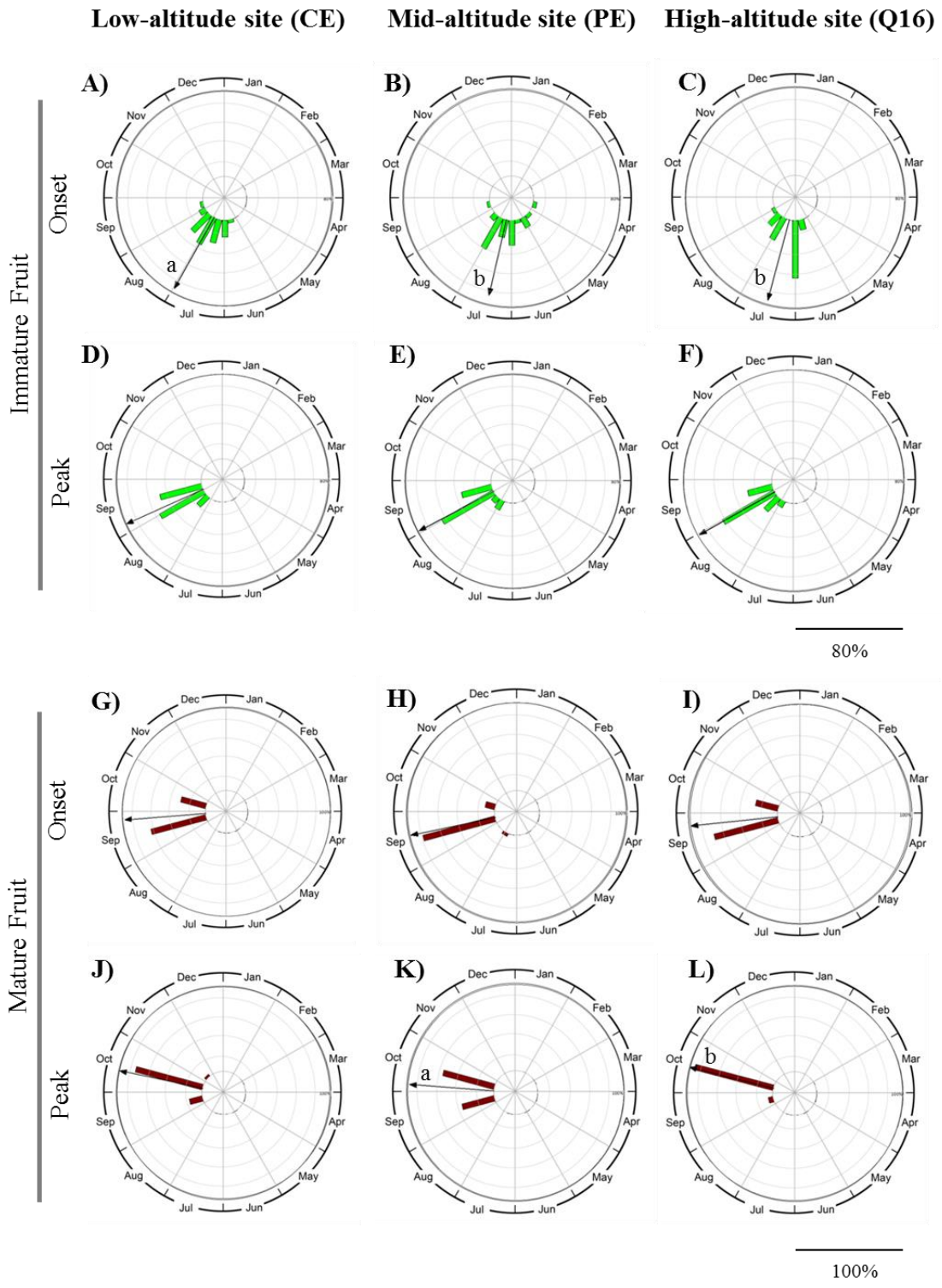

Fig.5. Circular distribuition of onset and peak dates for immature fruits **(A-F)** and mature fruits **(G-L)** based on the mean pattern from January to December 2014 for *Trembleya laniflora* individuals sampled in the three *campos rupestres* sites, low- (CE), mid- (PE), and high-altitude site (Q16), at Serra do Cipó, southeastern Brazil. All mean angles are significant (Rayleigh test, $p < 0.001$) and are represented by the arrows. The length of the mean vector r (0 to 1) indicates how grouped the dates are around the mean angles. Different letters (a, b) indicate of significant differences (Watson-Willians test, $p < 0.05$).

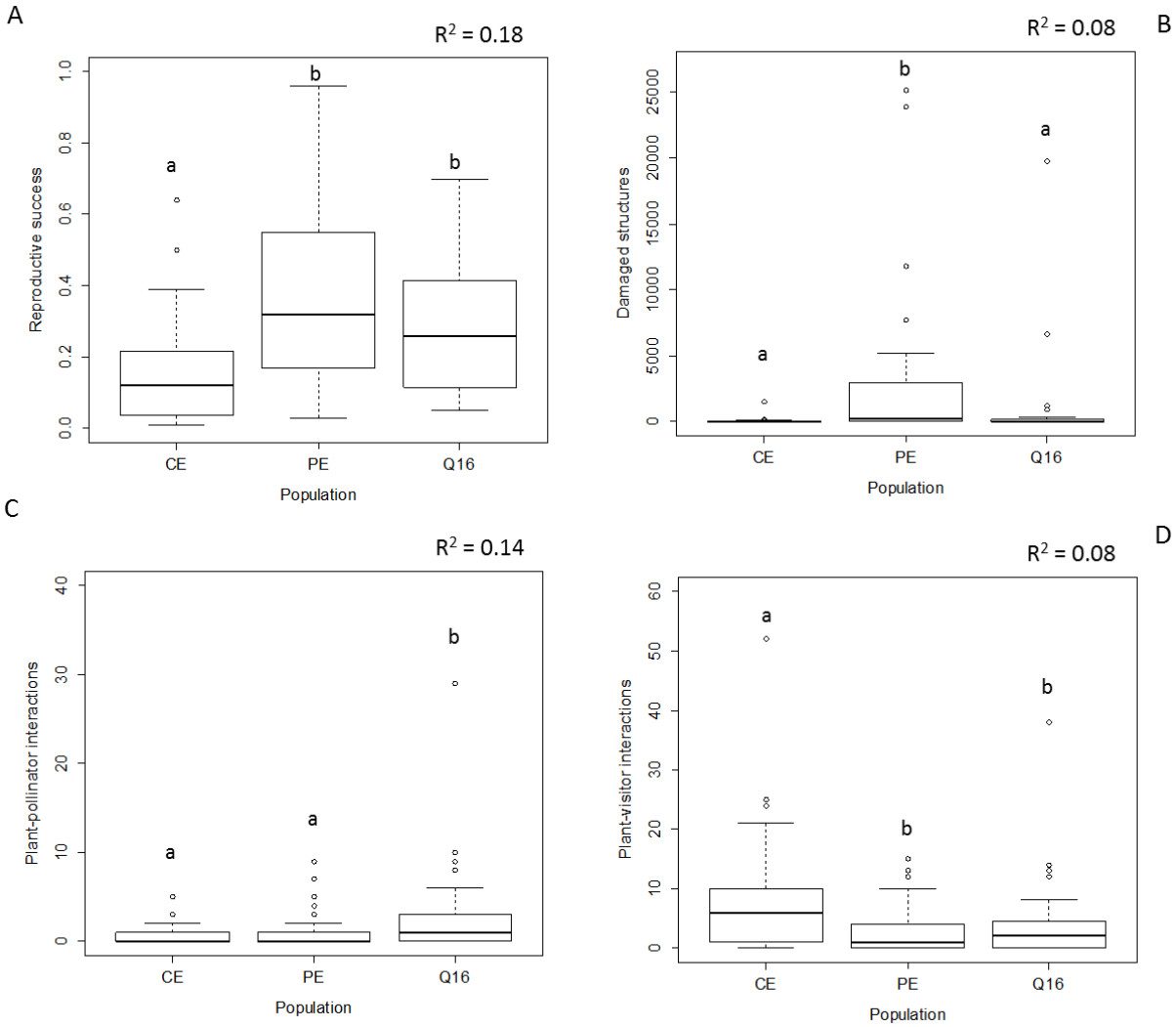

Fig.6. Reproductive success **(A)**, damaged structures **(B)**, illegitimate visits by floral visitors **(C)** and, legitimate visits by pollinator species **(D)** to *Trembleya laniflora* Cong. (Melastomataceae) individuals at low-altitude Cedro (CE), mid-altitude Pedra do Elefante (PE) and, high altitude Quadrante 16 (Q16) populations at Serra do Cipó (southeastern Brazil). Black lines on the boxes represent medians and different letters (a, b) indicate of significant differences (GLM, $p \leq 0.05$).

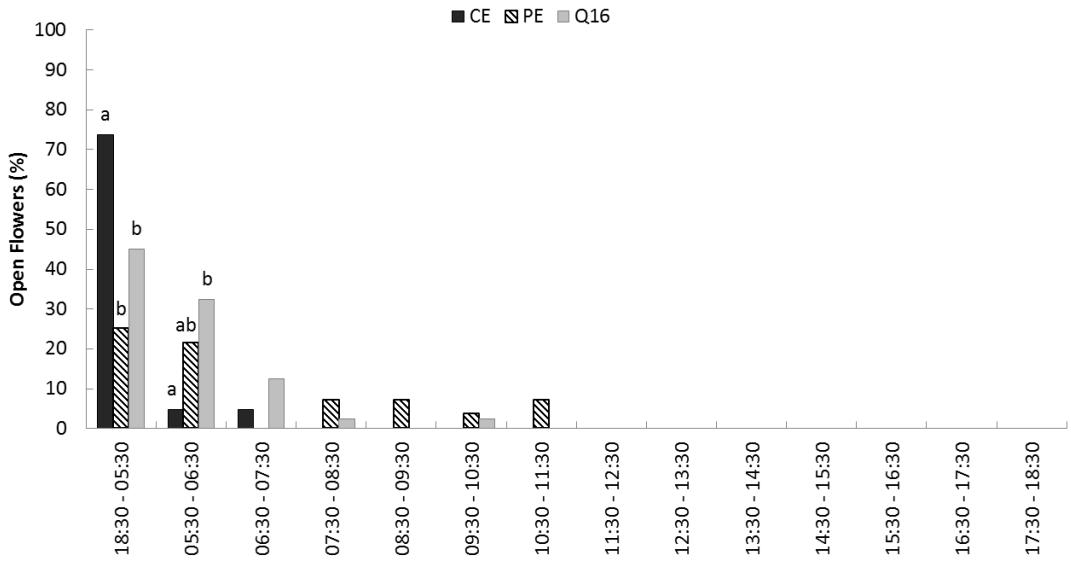

Fig.7. Timing of flower opening of *Trembleya laniflora* Cong. (Melastomataceae) in three sites, low-altitude Cedro (CE), mid-altitude Pedra do Elefante (PE) and, high-altitude Quadrante 16 (Q16), with distinct altitudes at Serra do Cipó, southeastern Brazil. Differed letters indicated significant differences (proportion test, $p<0.05$).

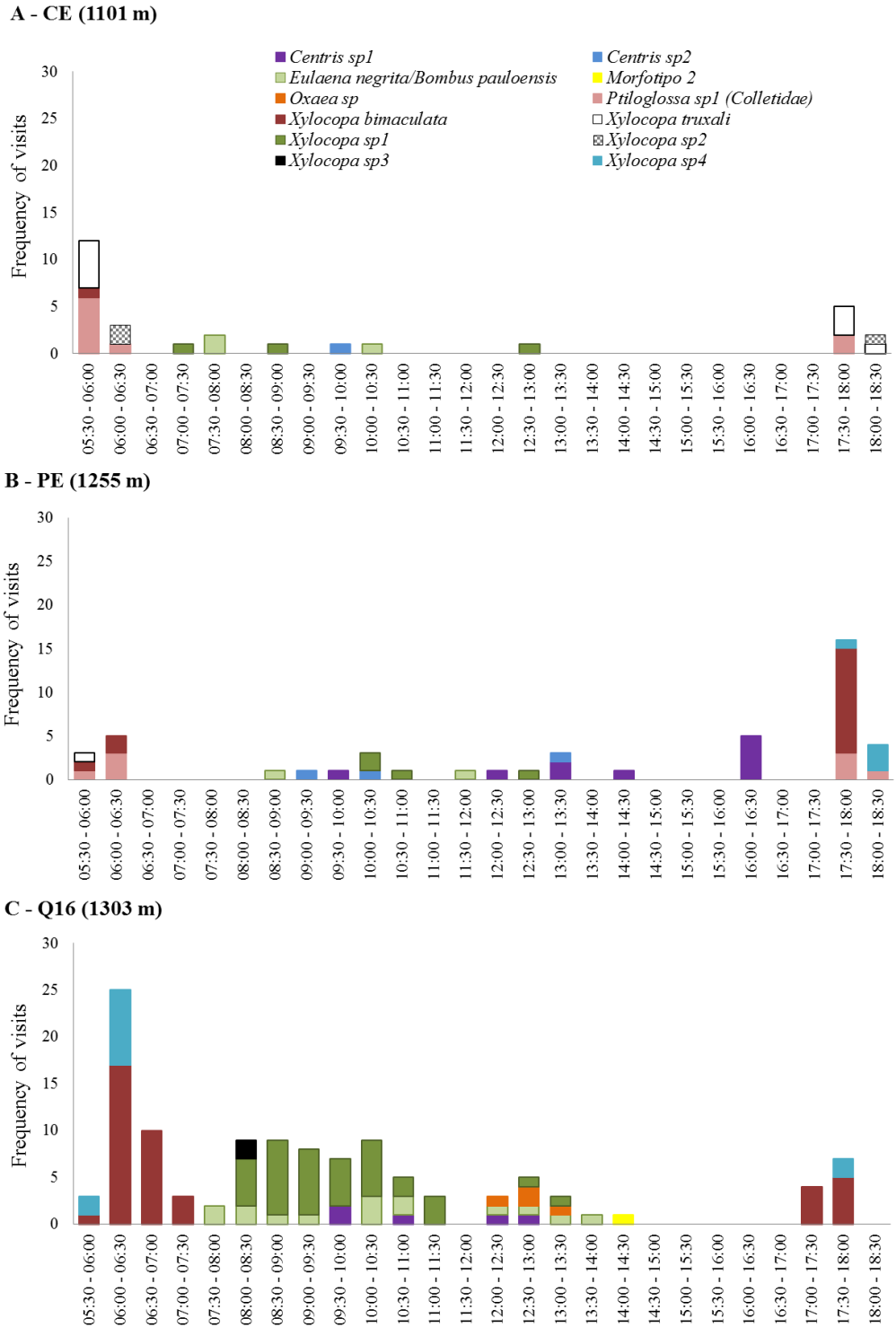

Fig.8. Frequency of legitimate visits by pollinators (principal and secondary) to flowers of *Trembleya laniflora* Cong. (Melastomataceae) in 29 individuals of low-altitude Cedro **(A)**, 28 individuals of mid-altitude Pedra do Elefante **(B)** and, 23 individuals of high-altitude Quadrante 16 **(C)** at Serra do Cipó (southeastern Brazil) (Quadrante 16 data are similar to Soares and Morellato, 2017).

8. SUPLEMMENTARY INFORMATION

8.1. Tables

Table S1. Results of circular statistical analyses testing for the occurrence of seasonality in phenological behavior at *Trembleya laniflora* (Melastomataceae) populations in *campo rupestre* at Serra do Cipó (Minas Gerais, Southeastern Brazil). Rayleigh test was performed for significance of the mean angle or mean date (*) and Watson-Willians test utilized for to evaluate the differences between the study sites (a and b letters). CE = low-altitude site (Cedro); PE = mid-altitude site (Pedra do Elefante) and, Q16 = high-altitude site (Quadrante 16).

	Onset_Floral Bud			Peak_ Floral Bud			Onset_Anthesis			Peak_Anthesis		
	CE	PE	Q16	CE [a]	PE	Q16 [b]	CE [a]	PE [b]	Q16	CE [a]	PE [b]	Q16 [b]
Number of Observations	31	31	31	31	31	31	31	31	31	31	30	31
Mean Vector (μ)	170.28°	163.68°	166.32°	198.46°	190.54°	184.69°	182.10°	169.85°	169.61°	215.48°	204.85°	200.96°
Mean Date	22-Jun	15-Jun	17-Jun	20-Jul	13-Jul	5-Jul	10-Jul	27-Jun	30-Jun	6-Aug	24-Jul	21-Jul
Length of Mean Vector (r)	0.94	0.92	0.93	0.94	0.93	0.95	0.93	0.91	0.95	0.96	0.93	0.93
Circular Standard Deviation	19.77°	23.45°	21.41°	19.60°	21.06°	19.02°	22.30°	24.20°	18.74°	17.06°	22.06°	21.80°
Rayleigh Test (Z)	27.52*	27.06*	26.96*	27.58*	27.96*	27.76*	26.64*	25.93*	27.85*	28.37*	25.87*	26.82*
	Onset_Immature Fruit			Peak_Immature Fruit			Onset_Mature Fruit			Peak_Mature Fruit		
	CE [a]	PE [b]	Q16 [b]	CE	PE	Q16	CE	PE	Q16	CE	PE [a]	Q16 [b]
Number of Observations	31	31	31	31	31	30	31	27	27	31	18	15
Mean Vector (μ)	210.12°	192.33°	195.08°	245.96°	242.22°	240.71°	265.88°	259.23°	262.88°	282.288°	275.21°	284.12°
Mean Date	1-Aug	14-Jul	17-Jul	6-Sep	3-Sep	1-Sep	26-Sep	20-Sep	23-Sep	13-Oct	6-Oct	15-Oct
Length of Mean Vector (r)	0.94	0.88	0.94	0.99	0.98	0.98	0.97	0.97	0.98	0.98	0.97	0.99
Circular Standard Deviation	19.82°	28.73°	20.60°	10.09°	12.72°	12.04°	13.88°	13.84°	12.31°	12.40°	14.34°	7.33°
Rayleigh Test (Z)	27.50*	24.11*	27.24*	30.05*	29.51*	28.70*	29.23*	25.47*	25.78*	29.58*	17.85*	14.76*

* $p < 0.001$ (Rayleigh test); Different letters $p < 0.05$ (Watson-Willians test)

Table S2. Spearman's correlation tests to the monthly local climate and among the monthly local climate and the monthly individual production of flower buds, flowers, immature and mature fruits of three study sites low-altitude CE, mid-altitude PE and, high altitude Q16 at Serra do Cipó, Espinhaço Mountain range, Southeastern Brazil. The measured environmental variables are photosynthetic active radiation (PAR, W/m2), precipitation (mm), temperature (ºC) and, relative humid (RH, %). *nc* represents no calculated values and *ns* indicates no significant value (p >0.05).

Sperman's Correlation	**PAR**	**Precipitation**	**Temperature**	**Relative humidity**
PAR	nc	0.34	0.62	-0.41
Precipitation	0.34	nc	0.69	0.34
Temperature	0.62	0.69	nc	0.36
Relative humidity	-0.41	0.34	0.36	nc
Flower bud	-0.11	-0.28	-0.42	-0.04 (ns)
Anthesis	-0.11	-0.27	-0.38	0.06
Immature fruit	0.09	-0.15	-0.24	-0.28
Mature fruit	0.42	0.47	0.24	-0.06

The ρ values are significant to p< 0.05 (Spearman's correlation test).

Table S3. Kruskall-Wallis test results to the comparison of the production of reproductive structures (bud flower, flower and fruits) by individuals of *Trembleya laniflora* (Melastomataceae) localized at low-altitude Cedro (CE), mid-altitude Pedra do Elefante (PE) and high-altitude Quadrante 16 (Q16) populations at Serra do Cipó, Espinhaço Mountain range, Southeastern Brazil. Values represent Kruskal-Wallis chi-squared, followed by p values in parenthesis.

	Bud flower	**Flower**	**Immature fruit**	**Mature fruit**
Monthly total production	0.08 (*0.96*)	0.31 (*0.85*)	0.56 (*0.76*)	1.33 (*0.51*)
Individual production	1.60 (*0.45*)	0.67 (*0.71*)	5.07 (*0.08*)	2.21 (*0.33*)
Maximum production	0.68 (*0.71*)	0.15 (*0.93*)	2.87 (*0.24*)	2.70 (*0.26*)

Table S4. Floral longevity (flower lifespan days) of *Trembleya laniflora* in three study sites, low-altitude Cedro (CE), mid-altitude Pedra do Elefante (PE) and, high-altitude Quadrante 16 (Q16), at Serra do Cipó, Espinhaço Mountain range, Southeastern Brazil. FB represents the sampled flower buds (42 in the CE, 28 in the PE, and 40 in the Q16 site).

	Flower lifespan (days)		
	CE (1101m)	**PE (1255m)**	**Q16 (1303m)**
FB1	No opened	3	3
FB2	4	4	3
FB3	3	4	3.5
FB4	No opened	4	3
FB5	3	4	2
FB6	3	4	3
FB7	3	4	2
FB8	3	No opened	3
FB9	3	4	3
FB10	3	4.5	3
FB11	No opened	4	3
FB12	3	4	3
FB13	2.5	4	3
FB14	2.5	4.5	2
FB15	3	4	4
FB16	3	4.5	No opened
FB17	3	4.5	2
FB18	2.5	4.5	2.5
FB19	3	5	3
FB20	3	4.5	3
FB21	3	4	2
FB22	3	4	3
FB23	3	4	2.5
FB24	3	3.5	No opened
FB25	3	4	3
FB26	3	4	2
FB27	3	4.5	3
FB28	3	4.5	2
FB29	3		3
FB30	2.5		3
FB31	3		3
FB32	2		3
FB33	3		3
FB34	2.5		3
FB35	3		3
FB36	2		2
FB37	No opened		2
FB38	3		2
FB39	3		3
FB40	3		4
FB41	No opened		
FB42	3		
Mean	**2.9**	**4.1**	**2.8**

Table S5. Total number and frequency (%) of illegitimate visits by non-pollinating visitors (flower visitor, pollen robber, and pollen thief) to flowers of *Trembleya laniflora* Cong. (Melastomataceae) in individuals of low-altitude Cedro (CE - 1101m), mid-altitude Pedra do Elefante (PE - 1255m) , and high-altitude Quadrante 16 (Q16 - 1303m), populations at Serra do Cipó, southeastern Brazil. Highlighted names represents the more frequent flower visitor species. N indicates the number of flowered individuals observed in each site.

	CE (1110m) N = 29		**PE (1255m) N = 28**		**Q16 (1303m) N = 23**	
Flower visitors	**Total**	**Freq. (%)**	**Total**	**Freq. (%)**	**Total**	**Freq. (%)**
Apis mellifera (Linnaeus)	2	0.64	4	3.31	4	2.90
Augochloropsis sp1	5	1.60	0	0.00	0	0.00
Augochloropsis sp2/*Augochloropsis* sp3	6	1.92	1	0.83	4	2.90
Augochloropsis sp4	1	0.32	1	0.83	7	5.07
Augochloropsis sp5	2	0.64	0	0.00	5	3.62
Augochloropsis sp6	0	0.00	2	1.65	1	0.72
Coleptera sp1	0	0.00	6	4.96	2	1.45
Coleptera sp2	0	0.00	0	0.00	1	0.72
Dialictus sp3	0	0.00	0	0.00	2	1.45
Diptera	0	0.00	1	0.83	0	0.00
Euglossa cf. *melanotricha* (Moure, 1967)	0	0.00	0	0.00	1	0.72
Euglossa sp1	0	0.00	0	0.00	1	0.72
Euglossa sp2	0	0.00	0	0.00	1	0.72
Exomalopis sp1	2	0.64	4	3.31	0	0.00
Exomalopis sp2	1	0.32	2	1.65	0	0.00
Formicidae sp2	0	0.00	3	2.48	0	0.00
Formicidae sp3	0	0.00	1	0.83	0	0.00
Hymenoptera sp1	4	1.28	1	0.83	0	0.00
Hymenoptera sp2	0	0.00	0	0.00	1	0.72
Hymenoptera sp3	0	0.00	0	0.00	1	0.72
Melipona trinofaciata	0	0.00	2	1.65	**107**	**77.53**
Neophasiphae sp.	0	0.00	2	1.65	0	0.00
Paratrigona sp	9	2.88	0	0.00	0	0.00
Polybia sp	4	1.28	0	0.00	0	0.00
Synoeca surinama (Linnaeus)	9	2.88	0	0.00	0	0.00
***Trigona spinipes* (Fabricius, 1793)**	**268**	**85.60**	**91**	**75.21**	0	0.00

8.2. Figure

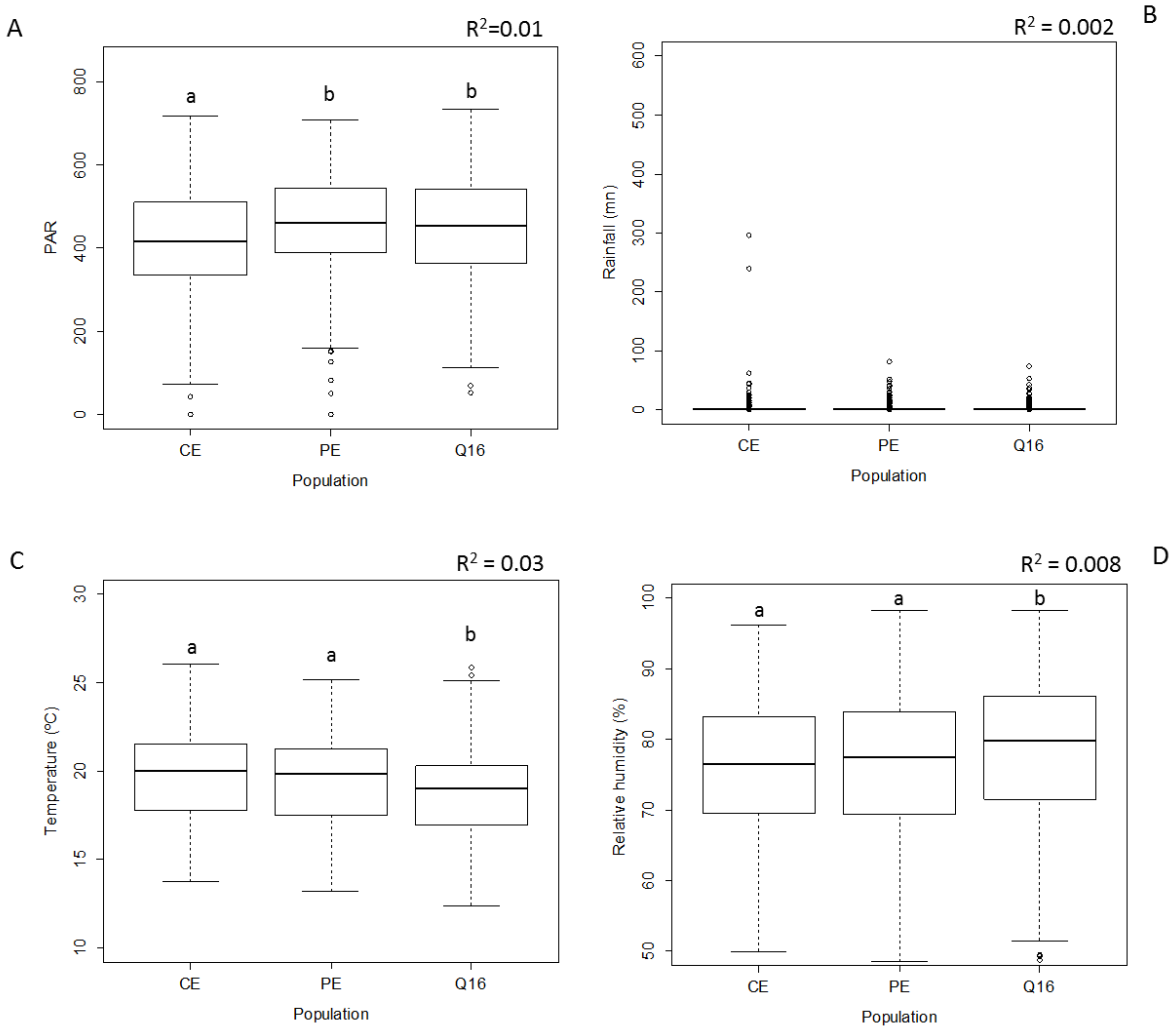

Fig.S1. Differences on monthly average of the environmental conditions of photosynthetic active radiation (PAR – W/m^2) **(A)**, precipitation (rainfall - mn) **(B)**, temperature (ºC) **(C)**, and relative humid (%) **(D)** between the study sites low-altitude Cedro (CE), mid-altitude Pedra do Elefante (PE) and high-altitude Quadrante 16 (Q16) of Serra do Cipó, southeastern Brazil. The black lines on boxes represent medians and different letters (a, b) indicate of significant differences ($p< 0.05$). All the GLMs were significant to $p < 0.001$. Data obtained to the 2014 year from climate stations (HOBO U30 Station GSM-TCP Model) localized at each study site.

Capítulo 3

Individual-level generalization increases reproductive success in a highly specialized pollination system

Chapter 3 - Individual-level generalization increases reproductive success in a highly specialized pollination system

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ABSTRACT

Conspecific individuals within populations differ in the use of available resources, including their association with pollinators. Plant functional traits, especially phenology, affect the structure of the plant-pollinator individual networks, and may determinate plants' reproductive output. However, no investigation has addressed the determinants of the structure of individual-level plant-pollinator networks in specialized pollination systems. Moreover, the roles of individuals within plant-pollinator networks and how it relates to plant reproductive success has been hardly investigated. Here, we aim to evaluate the variation in temporal (flowering phenology) and pollination (plant-pollinator interactions) resources use by individuals of *Trembleya laniflora* Cong. (Melastomataceae), a species with high specialized pollination system by crepuscular bees. We tested the effects of differences in resource use at the individual and population levels on reproductive output and success. We recorded phenological (time, amplitude, duration, and synchrony of flowering) and plant-pollinator interaction (number of flowers visited by pollinators and the richness of pollinators visiting flowers) parameters of individuals from three populations along an altitudinal gradient. To quantify specialization or generalization in flowering time and pollinator, we respectivelly adapted the IS index of individual specialization to phenological data and calculated the individual- and network-level specialization indices (d' and H_2'). Generalized Linear Models were used to assess the effect of individual and population-level specialization in flowering activity and pollinator interactions on plant reproductive success. *Trembleya laniflora* populations were generalist – high IS - in

their phenological activity (i.e., high interindividual temporal overlap), but highly specialists in their interactions with pollinators (i.e., high network-level specialization). Population with highest reproductive success have individuals showing greater generalization in flowering activity (high synchrony) and in interactions with pollinators (higher frequency of visits, connectance, evenness and low network-level specialization). At the individual level, higher flowering syncrony and centrality in the pollination networks were associated with higher reproductive success. We conclude that phenological generalization and higher synchrony incresed the reproductive success of the three populations of *Trembleya*. The highly specialized pollination system of *Trembleya lanifora* benefit from generalist individuals regarding the use of time and pollinators, and may be the key evolutionary feature of this narrow endemic species from rupestrian grasslands outcrops.

Keywords: Individual-level ecological networks, flowering overlap, synchrony, rupestrian grassland

1. INTRODUCTION

Conspecific individuals within populations differ in the use of available resources (eg. preys, soil nutrients, host plants, pollen, pollinator services), and this intraspecific variation has an important role in many ecological processes (Bolnick et al., 2003, Araújo et al., 2010, 2011; Bolnick et al., 2011; Dall et al., 2012; Wolf and Weissing, 2012; Zywiec et al., 2012; Tur et al., 2014). Recent studies have pointed out the importance of intraspecific trait variations to the structure and function of plant populations and communities (Jung et al., 2010; Violle et al., 2012; Siefert et al., 2015; Kuppler et al., 2016), and individual variation has been also observed for plant interactions, including pollination (e.g. Dupont et al., 2011; Tur et al., 2014). Because plant-pollinator interactions are mediated by flower traits (eg. Stang et al., 2006; Zywiec et al., 2012) and individuals are the true actors in the interactions (Tur et al., 2014), individual-level variations in floral and vegetative traits could affect their interaction with pollinators, ultimately influencing the plant reproductive success (Gómez and Perfeccti, 2012; Kuppler et al., 2016) and adaptation in natural ecosystems (Bolnick et al., 2003; Gómez and Perfeccti, 2012; Kuppler et al., 2016).

Plant functional traits are defined as any morphological, physiological or phenological characters that are measurable at individual-level and impact fitness (Violle et al., 2007). Studies evaluating the role of plant intraspecific trait variations in the interaction with pollinators have mainly focused on morphological (eg. number of flowers, inflorescence height, corolla diameter, floral shape) and physiological (eg. floral scent, nectar volume and concentration) floral traits and pollinators (Herrera, 1995; Zywiec et al., 2012; Gómez et al., 2014a). However, phenology is also important to be considered as a functional reproductive trait. Phenological responses are known to vary among conspecific individuals (Newstron et al., 1994; Marquis, 1988; Sakai et al., 2005, Otárola et al., 2013) and spatial and temporal patterns of flowering overlap influence plant-animal interactions and mediate plant individual and populational fitness (Augspurger, 1981; Maroho, 2002; Dupont et al., 2014).

Time is considered as one niche dimension, and temporal segregation or agregation of flowering time is a response to distinct ecological pressures such as facilitation or competition for mutualists (Rathcke, 1983; Rathcke and Lacey 1985; Van Schaik et al., 1993; Staggemeier et al., 2010). Pollination systems can also be understood as another

niche dimension (Ambruster et al., 1994; Pauw, 2013; Gómez et al., 2014a) and the pollinators as the resources utilized by plants (Waser, 2006). Specialist and generalist species will differ in niche breadth and overlap (Gómez et al., 2014b). However, the high specialization and generalization only represent two extremes of a continuum in resource use by individuals or species (Waser, 2006). In the context of populations and taking individual variation into account (Bolnick et al., 2003), we can consider some specialist species as composed by generalist individuals using the majority or all the available resources of the population niche (time or pollinators). Individual generalization in phenology would impart higher temporal overlap among individuals in the population, increasing the availiability of potential mates. Moreover, aggregation of flowering times enhances attractiveness of the population to pollinators, increasing pollination success and, consequently, fruit set (Augspurger, 1981,1983; Rathcke, 1983).

The relationships between plant individuals in a population and their pollinators can be described as an ecological network (Fortuna et al., 2008). In self-incompatible and animal-pollinated species, the pattern of pollinator sharing among the individuals of a network has consequences to pollen transfer, mating and gene flow among individual plants (McDoanld, 2007; Gómez et al., 2011). In this way, how connected an individual is in the interaction network, is likely related to its reproductive success (Gómez and Perfeccti, 2012). Gómez and Perfectti (2012) showed that central and generalist individuals have higher reproductive success than peripheral and specialist individuals. In species-level ecological networks, more abundant and generalist species also hold central positions and are more important than peripheral, specialist species for the stability and functionality of the whole system (Mártin González et al., 2010, Sazima et al., 2010). Despite the recent advances on the key role of individual variation in many ecological processes (Bolnick et al., 2011, Dall et al., 2012, Tur et al., 2014, Guerra et al., 2017), few empirical studies have utilized ecological networks as a tool to explore plant-pollinator relationships at the individual-level (Gómez and Perfectti., 2012, Tur et al., 2014, Valverde et al., 2016). Most studies of individual-level ecological networks have considered generalized plant or animal species (Gómez and Zamora, 1999, Araújo et al., 2010, Gómez and Perffectti, 2012, Tur et al., 2015, Guerra et al., 2017). Here we propose to study the individual network of a specialized plant-pollination systems.

Trembleya laniflora Cong. (Melastomataceae) is a shrub that presents floral features (large, showy and white-colored flowers of nocturnal anthesis) associated with a highly specialized buzz-pollination system mediated by large crepuscular-foraging bees (Martins, 1997; Soares and Morellato, 2017). The endemic plant species is restricted to rocky outcrops of *campos rupestres* in the South of Espinhaço Mountain range, Southeast Brazil, and the naturally isolated populations differ in their phenology and other floral traits, as well as in their reproductive success (see Chapter 2). Here, we evaluated the intraspecific variation in flowering phenology and interactions with pollinators of *T. laniflora*, assessing how specialization at the individual and population levels relates to reproductive success. We quantified the reproductive phenology (amplitude, duration, and synchrony) and pollinator interactions (number of flowers visited by a pollinator) of *T. laniflora* individuals from three distinct populations. We further tested the effect of phenological and pollination specialization on plant reproduction. We predicted generalist individuals regarding the use of phenological (time) and pollination (pollinators) resources to have higher reproductive success in comparison to more specialized plants.

2. MATERIAL AND METHODS

2.1. Study system

This study was conducted in three isolated sites localized in the rupestrian grasslands at Serra do Cipó, south of Espinhaço Mountain Range (State of Minas Gerais, southeastern Brazil). The sites are distant at least 4 km (linear and planar distances) from each other, and at distinct altitudes, locally named: Cedro (CE), the low-altitude site at 1011 m (43º 34' 56" W; 19º 14' 16" S), Pedra do Elefante (PE), the mid-altitude site at 1255 m (43º 33' 20" W; 19º 17' 34" S) and the high-elevettion site Quadrante 16 (Q16); 43º 35' 31"W, 19º 17' 42" S) at 1303 m a.s.l. (Rocha et al., 2016). During 2014, at each site, we recorded the reproductive phenology and pollination interactions of at least 31 reproductive individuals of *Trembleya laniflora* Cogn. (Melastomataceae), all sampled and tagged inside one-hectare plots as described by Soares and Morellato (2017). *Trembleya laniflora* is a non-apomictic and self-incompatible endemic shrub, whose occurrence is restricted to rocky outcrops of the rupestrian grasslands of South Espinhaço range (Martins, 1997; Soares and Morellato, 2017).

2.2. Reproductive success

Phenological observations were realized monthly from January to December and the number of reproductive structures (floral buds, flowers, immature and mature fruits) was estimated by periodic counts of five tagged branches in the sampled individuals of *T. laniflora* (32 from CE, 32 from PE, and 31 Q16). During the main flowering season (July and August) the observations were done bi-weekly. Individual reproductive success was calculated as the ratio between the number of immature fruits and the total number of flowers produced.

2.3. Phenological individual specialization

The relative degree of individual specialization in phenology was estimated for individuals and populations of *Trembleya laniflora.* We employed the definitions of specialization based on Bolnick et al. (2002), here modified to the context of phenological research. We adapted the metrics to take in account temporal autocorrelation in all steps of the analysis. That was necessary since the occurrence of one phenological event, for instance, production of flowers, is not independent of the previous flower bud production, as well as fruit production is dependent of the previous flowering event. At the individual-level, we calculated the PS_i - Proportional Similarity index (*sensu* Bolnick et al., 2002), which measures the similarity in the use of phenological time between an individual *i* and the overall population (studied site). The PSi values represent the proportional use of the flowering time by each individual compared to the phenological curve of its population (i.e. overlap among the individual phenology and the population phenology). Therefore, the period (duration) and intensity (amount) of flower production (flowering activity) over time were taken into account. For each individual plant the degree of specialization was calculated as:

$$\mathrm{PSi} = 1 - 0.5 * (\sum_{j=1}^{n} |pij - qj|)$$

where p_{ij} is the proportion of flowers produced during the *j*th month in relation to the total production of individual *i*, q_j is the proportional production of the population in the *j*th month and *n* is the phenophase duration of the population (in months). To minimize potential effects of crown size or plant height, q_j was calculated as the average of p_{ij}

values instead of summing up the numbers of flowers in month *j* across all individuals (total flowers counted in each census) and dividing it by the grand total (total flowers counted in all censuses). For this analysis, the bi-weekly flower counting in the flowering season (July and August) were pooled into one sampling per month. At the population level, we calculated the IS index of individual specialization (Bolnick et al., 2002), which is the average of PSi values calculated across all individuals. Both PSi and IS assume the value 1 when there is no individual specialization and lower decimal values as individual specialization becomes stronger.

We tested the significance of the phenological specialization for *T. laniflora* populations using null model analysis. Some assumptions about phenological patterns were made when testing the observed IS values against a null distribution of IS values generated by Monte-Carlo resampling (999 simulations): for each individual (i) the onset of flowering was randomized and dependent on the individual flowering duration being sorted within the interval when the population is flowering (Soares and Morellato, 2017; see also Chapter 2) which is assumed to be the physiological limit of population response; (ii) due to the temporal autocorrelation of the phenology data, the shape of the phenological curve was kept immutable during the randomizations (see Staggemeier et al., 2010). The PSi and IS were recalculated for each simulation and the significance of observed IS was estimated by the number of times that the observed value was higher or lower than the simulated values (two-tailed test). Phenological specialization occurred when the observed IS was lower than at least 975 of the simulated values; or phenological generalization occurred when IS was higher than at least 975 of the simulated values. The analyses were conducted in the R language (Ross and Gentleman, 1996) and a script with the analytical steps is available in the supplementary information (Appendix 1).

2.4. Diversity of pollinators and frequency of visits

During the main flowering season (July and August), we randomly conducted 10-minute surveys of floral visitors to blooming individuals of the studied sites (37 from low-CE, 35 from mid-PE, and 32 from high-Q16). All observations were carried out from 05:30h to 18:30h, totalizing 80 hours per population. We quantified the available resources (number of open flowers), the number and identity of bee pollinator species, and the number of visited flowers by each bee species. Pollinator species were mainly

identified in the field and when necessary collected for subsequent identification (see Soares & Morellato, 2017 and Table S1, supplementary information). Only the plant individuals surveyed for at least 6 censuses (total of 1 hour) were considered for the analyses below.

2.5. Network Metrics

To evaluate and compare the plant-pollinator interactions between and within *T. laniflora* populations we constructed individual-based plant-pollinator networks for each studied site. We considered as visits as the number of flowers of each individual visited by pollinators. To draw the networks we used the number of visits partitioned by number of surveys in each plant (time of individual observation). For each network we calculated the network-level metrics: total number of interactions (I), connectance (C) (Jordano, 1987), interaction evenness (E_2) (Tylianakis et al., 2007), and network-specialization index (H_2'); and the individual-level metrics: centrality (degree, closeness, and betweenness) and individual-specialization index (d') (Blüthgen et al., 2006). We compared the structural properties of the individual-based networks (Blüthgen et al., 2006, Dormann et al., 2008, Tur et al. 2014).

Connectance (C) is the proportion of the number of observed interactions (I) in relation to the number of possible interactions in each network (Jordano, 1987). Interaction evenness (E_2) measures the heterogeneity of interaction frequencies (symmetry). This metric varies from 0 to 1, with lower values indicating uneven and higher values indicating uniform distribution of links in the networks (Tylianakis et al., 2007). Network-level specialization (H_2') was used to quantify the degree of specialization of the entire population. H_2' is a network-level measure of specialization, based on the deviation of a node' realized number of interactions and that expected from each node' total number of interactions, and related to the interaction diversity (H_2), i.e. Shannon's diversity of interactions for the whole network. H_2' values vary from 0 (no specialization) to 1 (perfect specialization for given interactions totals) (Blüthgen et al., 2006; see also Dormann et al., 2009).

Centrality metrics - degree, closeness and betweenness - were utilized to evaluate how well connected is one individual plant in the network (Gómez and Perfectti, 2012). Degree centrality (Cd) asseses the importance of a node (here, *T. laniflora* individual) to

its normalized degree (number of links) in the network, and it is defined by the number of links that are connected to a determined node. Closeness centrality (Cc) measures the proximity of a node to all the nodes in the network, and represented, therefore, how close a plant individual is to all the individuals in the population. Addictionally, betwenness centrality (Cb) measures the importance of a node as a connector between the parts of the network (Freeman, 1979). Generalist nodes tend to be located in the most central positions in ecological networks, therefore, the centrality metrics were also used to evaluate the degree of specialization of individuals (Martin-González et al., 2010, Sazima et al., 2010; Dormann, 2011; Gómez and Perfectti., 2012). Finally, the individual-level specialization index (d') was used to quantify the degree of interaction specialization of *T. laniflora* individuals. The d'- index represents the deviation of the observed interaction frequencies from a nulll model which assumes that all partners (plant individuals and pollinators) are used in proportion to their availability. This index varies from 0, indicating generalization to 1, indicating higher specialization (Blüthgen et al., 2006). All metrics were calculated utilizing the package *bipartite* in R (Dormann et al., 2008) and the significance of network level metrics was obtained by comparison with null networks generated through the function *vaznull*, which constrains the connectance, number of interactions and network size (Vázquez et al., 2007).

2.6. Data analyses

To evaluate the flowering synchrony and compare the temporal phenological niche overlap between *T. laniflora* individuals and their populations, we calculated the individual (Xi) and population (Z) flowering synchrony indices (Augspurger, 1983). Xi represents the synchrony of a given individual to its population and Z is the synchrony for the entire population (average Xi). Both vary from 0 (no synchrony) to 1 (perfect synchrony; Augspurger, 1983). Differences in the Xi values among populations were tested through a Generalized Linear Model (GLM) with sites as predictor and Xi values as response variable (Nelder and Wedderburn, 1972). We also tested for differences between populations in the (i) individual-level phenological specialization (PSi), (ii) total number of flowers visited per census; and (iii) reproductive success using GLMs with the populations (sites) as the predictor variable (Nelder and Wedderburn, 1972).

To assess the importance of the phenological and interaction specialization in the reproductive sucess of *T. laniflora* individuals, we generated GLMs with (iv)

phenological specialization (PSi), (v) individual synchrony (Xi), (vi) number of flowers, (vii) total number of visits standartized by number of censuses, vii) individual-level interaction specialization (d'), and viii) centrality (Cd, Cc and Bc) as predictors and reproductive success as the response variable. Effects of the phenological specialization (PSi), individual synchrony (Xi), number of flowers, and visits on individual-level interaction specialization (d') were also tested with GLMs (Nelder and Wedderburn, 1972). We assumed Gaussian error distribuition to all the GLMs. The analyses were conducted in R (R Development Core Team, 2011).

3. RESULTS

The three populations of *Trembleya laniflora* showed high flowering synchrony ($Z \geq 0.68$), with significantly higher population synchrony observed in the mid-altitude PE compared to high- Q16 and low-altitude CE populations (Table 1). However, the identity of the population offered a weak explanation for *T. laniflora* individual synchrony (Xi model; $R^2 = 0.05$; Figure S1A, supplementary information). The number of visits by pollinators was also weakly influenced by the identity of the population (p=0.02, R^2= 0.09).

The populations differed in the reproductive success, with low-altitude CE individuals showing the lower success compared to mid- PE and high-altitude Q16 (R^2=0.26, p <0.001) (Figure 1). We did not observe differences between populations in phenological specialization (PSi model, R^2=0.02, p=0.34; Figure S1B, supplementary information). All populations presented significantly low individual specialization in flowering activity (IS values higher than expected by chance), with the lowest phenological specialization observed in the low-altitude CE population (Table 1, Figure 2). In contrast, plant populations were significantly and highly specialized in their interactions with the pollinators (Table 1).

We found more visits by pollinators (I), higher connectance (C), more evenly distributed interaction (E_2) and lower network-level specialization (H_2') in the Q16 population, in comparison to PE and CE (Table 1, Figure 3). The reproductive success of *T. laniflora* individuals were related to their centrality degree in the pollination networks and the total number of visits was negatively related to individual–level specialization (d') (Table 2).

4. DISCUSSION

Our results demonstrate the importance of the generalist individuals and populations to the reproductive success of a restricted endemic species with a highly specialized pollination system. At the population level, higher flowering synchrony and network level generalization were associated to higher reproductive success. At the individual level, greater degree of centrality (higher generalization in terms of number of partner pollinator species) was associated with higher reproductive success (see also Gómez and Perfeccti, 2012). Our findings support the idea that high generalization in plant-animal interactions can benefit reproduction.

Flowering synchrony and number of visits by pollinators were weakly explained by population identity suggesting the intraspecific variations observed in the interaction networks and reproductive success were mediated by other features of the plant individuals, and not only their spatial localization (see Chapter 2). We found a higher reproductive success on mid- PE and high-altitude Q16 populations, composed by more synchronic and no specialized individuals in their flowering phenology. Synchronic flowering among conspecifics are described to enhance attractiveness of plants to pollinators, influencing the individual and populational reproductive success (Augspurger, 1981; Rathcke, 1983; Rathcke and Lacey, 1985; Dupont et al., 2014). Visitation by pollinators are sensitive to floral density (Carstensen et al., 2014), so the number of plant-pollinator interactions, the pollen flow, and consequently, the reproductive success depend not only on individual availability of floral rewards but also on the floral display of the conspecifics (Augspurger, 1983; Rathcke, 1983; Marquis, 1988). Likewise, in ecological individual-level networks, individual roles such as the centrality and generalization in the networks can be affected by floral traits of conspecific individuals (Krause et al., 2009; Gomez and Perfeccti., 2012). Therefore, the higher reproductive success observed on *Trembleya laniflora* populations composed by no specialized individuals in their phenology migth be determined not only by responses of the focal individuals but also by phenological traits of conspecific individuals that interact in the same network.

Here, the generalizations in the flowering responses had beneficial effects on the reproductive success of the *Trembleya laniflora* populations. Soares and Morellato (in prep., see Chapter 2) also observed positive effects of phenological traits such as long

anthesis duration (in months) and high production of flowers on the production of mature fruits and consequent reproductive success of *T. laniflora*. We consider the phenological traits described as responses to strong selective pressures on the reproductive biology of the species leading to a high reproductive success. Although at the individual level we have not found significant effects of the low phenological specialization and high flowering synchrony on reproductive success, low specialization in the offer of floral resources to the pollinators demonstrated to have a key role in the reproductive output of *T. laniflora* populations.

In addition to the beneficial effects of low degree of individual specialization in phenological activity, we also found a high reproductive success in the population with the highest number and frequency of visits, more symmetric network and lower network-level specialization (H_2'). The individual-level specialization (d') was also negatively affected by the amount of interactions, so that plant individuals that were more specialized in their interactions were less visited by bee species. In the others words, populations composed by more generalist individuals that interact more with their pollinators have higher reproductive success. Tur et al., (2013) suggest that animal-pollinated species which are highly dependent on insects to the their seed set tend to be more central and connected in networks. Similarly, we found a significant effect of the degree of centrality of individuals on reproductive success of *T. laniflora*. In this way, more central individuals in the networks and more generalist in their interactions with the pollinator species had reproductive advantages in this pollinator-dependent plant species, and, therefore, the generalization in the pollination interactions also demonstrated to be advantageous to their reproduction.

In species-level ecological networks central, strongly linked nodes are described to have a major impact on community structure and function (Martin-González et al., 2010; Sazima et al., 2010). Correspondingly, in individual-level networks generalist individuals occupying more central positions and presenting greater diversity of interactions are important to mantain the plant-pollinator interactions and have a fundamental role in the maintenance of natural populations, even in species presenting highly specialized pollination system as the case of *Trembleya laniflora* (Gómez and Perfectti, 2012). High diversity and low specialization degree in the ecological interactions of individual plants positively influence the stability of the population and

ensure a higher reproductive success, mainly in endangered environments (Waser et al., 1996; Hooper et al., 2005; Violle et al., 2012; Kuppler et al., 2016).

We conclude that even in specialized pollination systems, generalist responses of flowering and pollination have a key role in plant-pollinator interactions and a positive effect on plant reproductive success. The knowledge on individual-level interaction networks is based mostly on generalist pollination systems (e.g. Dupont et al., 2011; Gómez et al., 2011; Gómez and Perfetti, 2012; Valverde et al., 2016, Kuppler et al., 2016) and here we observed that more generalist and central individuals in networks have higher success also in a specilized pollination system. Moreover, phenological generalization and higher synchrony were also beneficial to the reproductive success of the three *Trembleya* populations. The specialization by plants on pollinators can be a variable attribute of populations and individuals rather than a functional trait of species (Herrera, 2005; Gómez and Perfeccti, 2012). The scarcity of studies of individual-level networks on these specilized pollination systems may limit our conclusions, but plant species such as *T. laniflora*, with a highly specialized pollination system may benefit from generalist individuals regarding the use of time and pollinators, as comproved by the low individual specialization in the flowering activity and higher reproductive success of the more central individuals in the pollination networks.

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7. TABLES AND FIGURES

7.1. Tables

Table 1. Relative degree of phenological specialization and synchrony in flowering phenology and network metrics in three populations of *Trembleya laniflora* Cong. (Melastomataceae) from rocky outcrops at Serra do Cipó rupestrian grasslands, Southeastern Brazil. IS = Individual Specialization index in flowering activity, Z = Population synchrony index, CE = low-altitude site (Cedro); PE = mid-altitude site (Pedra do Elefante) and, Q16 = high-altitude site (Quadrante 16). All the H_2' values are significant ($p < 0.05$) based in null models generated by 9999 repetitions. All the IS values are significant ($p < 0.05$) based in null model generated by 999 repetitions (Monte Carlo test). Letters (a, b) indicate significant differences at $p<0.001$.

	CE	**PE**	**Q16**
Population Synchrony (Z)	0.68^b	0.75^a	0.72^b
Phenological specialization (IS)	0.65	0.61	0.59
Network metrics			
Total number of interactions (I)	93^b	66^b	419^a
Connectance (C)	0.17	0.23	0.31
Interaction evenness (E_2)	0.53	0.51	0.67
Specialization network-level (H_2')	0.92	0.64	0.40

Table 2. Summary results of Generalized Linear Models (GLM) showing the effect of flowering overlap (individual-level phenological specialization degree - PSi), individual flowering synchrony (Xi), floral resources availability to the pollinators (average number of open flowers), plant-pollinator interactions range (number of flowers visited by pollinators), and centrality metrics, degree (Cd), closeness (Cc) and, betweenness (Bc), on the individual-level specialization index (d') and reproductive success of individual plants of *Trembleya laniflora* (Melastomataceae). Significant values ($p < 0.05$) are shown in boldface; nc = no calculated values.

	d' value		**Reproductive success**	
	t	***p***	**t**	***p***
Flowering activity overlap (PSi)	0.37	0.71	0.47	0.63
Individual synchrony (Xi)	0.34	0.73	1.32	0.19
Number of flowers	1.58	0.12	-0.69	0.49
Plant-pollinator interactions range	**-2.62**	**0.01**	0.11	0.91
Individual-level specialization (d')	nc	nc	-0.61	0.54
Centrality degree (Cd)	nc	nc	**2.05**	**0.05**
Closeness (Cc)	nc	nc	0.95	0.34
Betwenness (Bc)	nc	nc	-1.33	0.19
$\mathbf{R^2_{model}}$		***0.18***		***0.32***

7.2. Figures

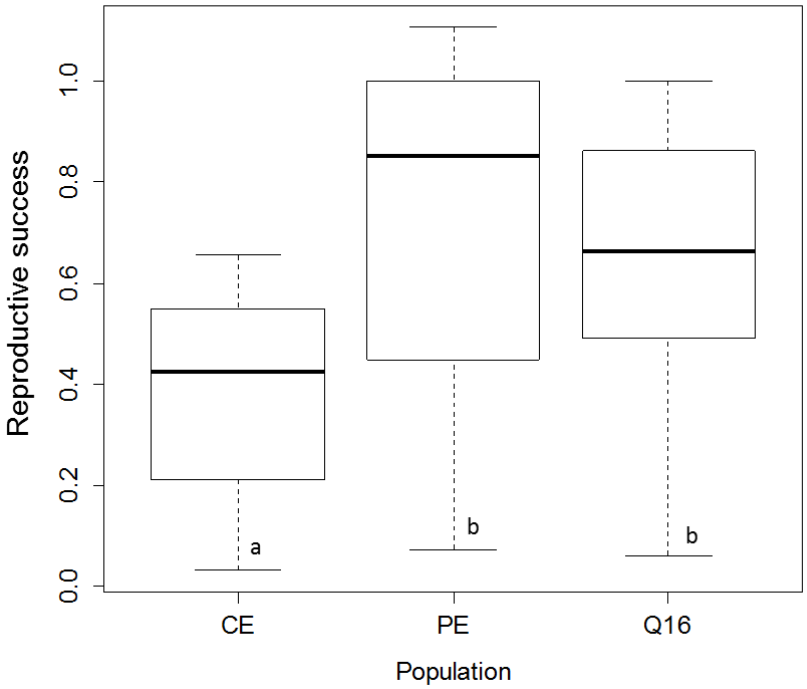

Fig.1. Proportion of immature fruits produced in relation to the amount of flowers offered (reproductive success) by individuals of three populations of *Trembleya laniflora* Cong. (Melastomataceae) from rocky outcrops at Serra do Cipó rupestrian grasslands, Southeastern Brazil. CE = low-altitude site (Cedro); PE = mid-altitude site (Pedra do Elefante) and, Q16 = high-altitude site (Quadrante 16). Different letters (a, b) indicate significant differences at $p<0.001$ values ($t_{PE}=5.68$; $t_{Q16}=5.06$; $R^2_{GLM}=38.3$).

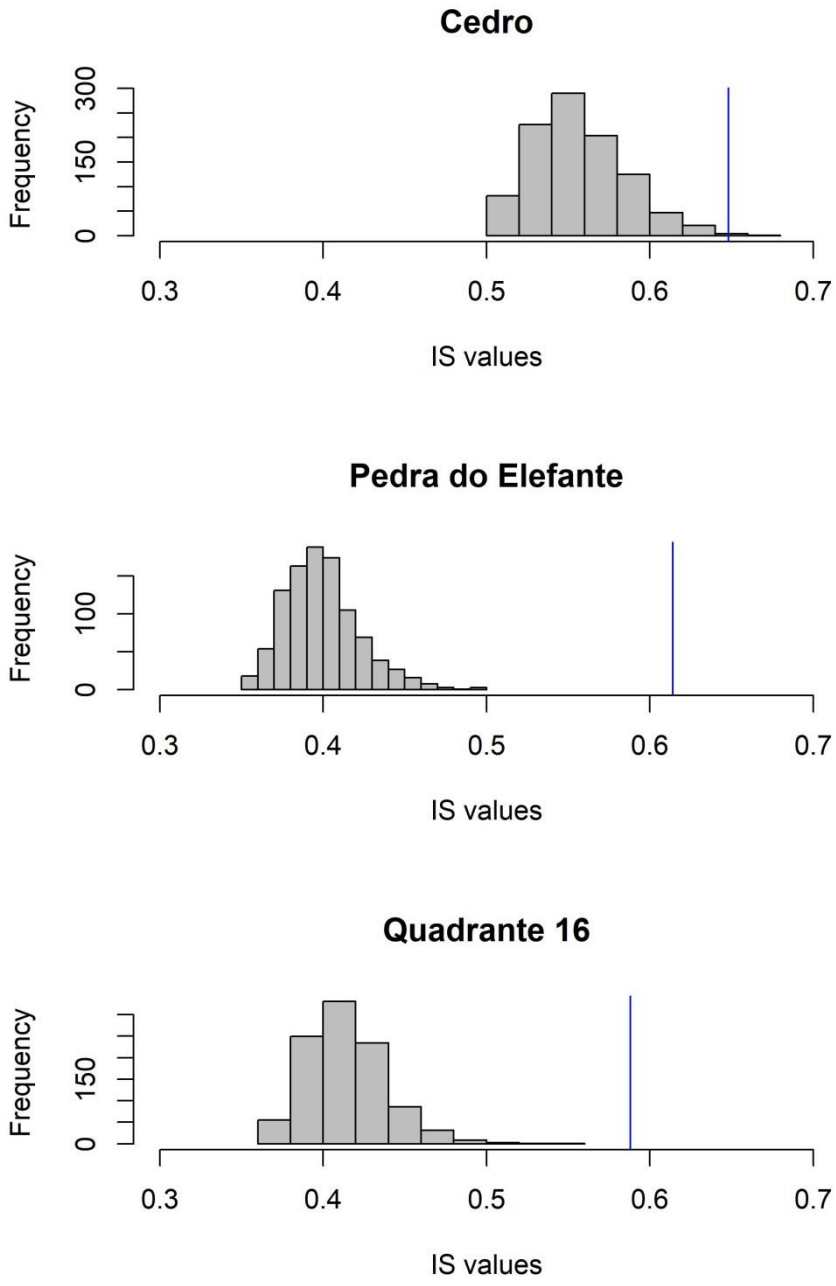

Fig.2. Observed (blue line) and distribution of simulated (grey bars) IS index measuring individual specialization in flowering activity in three populations of *Trembleya laniflora* Cong. (Melastomataceae) from rocky outcrops at Serra do Cipó rupestrian grasslands, Southeastern Brazil. CE = low-altitude site (Cedro); PE = mid-altitude site (Pedra do Elefante) and, Q16 = high-altitude site (Quadrante 16). IS assume the value 1 when there is no individual specialization.

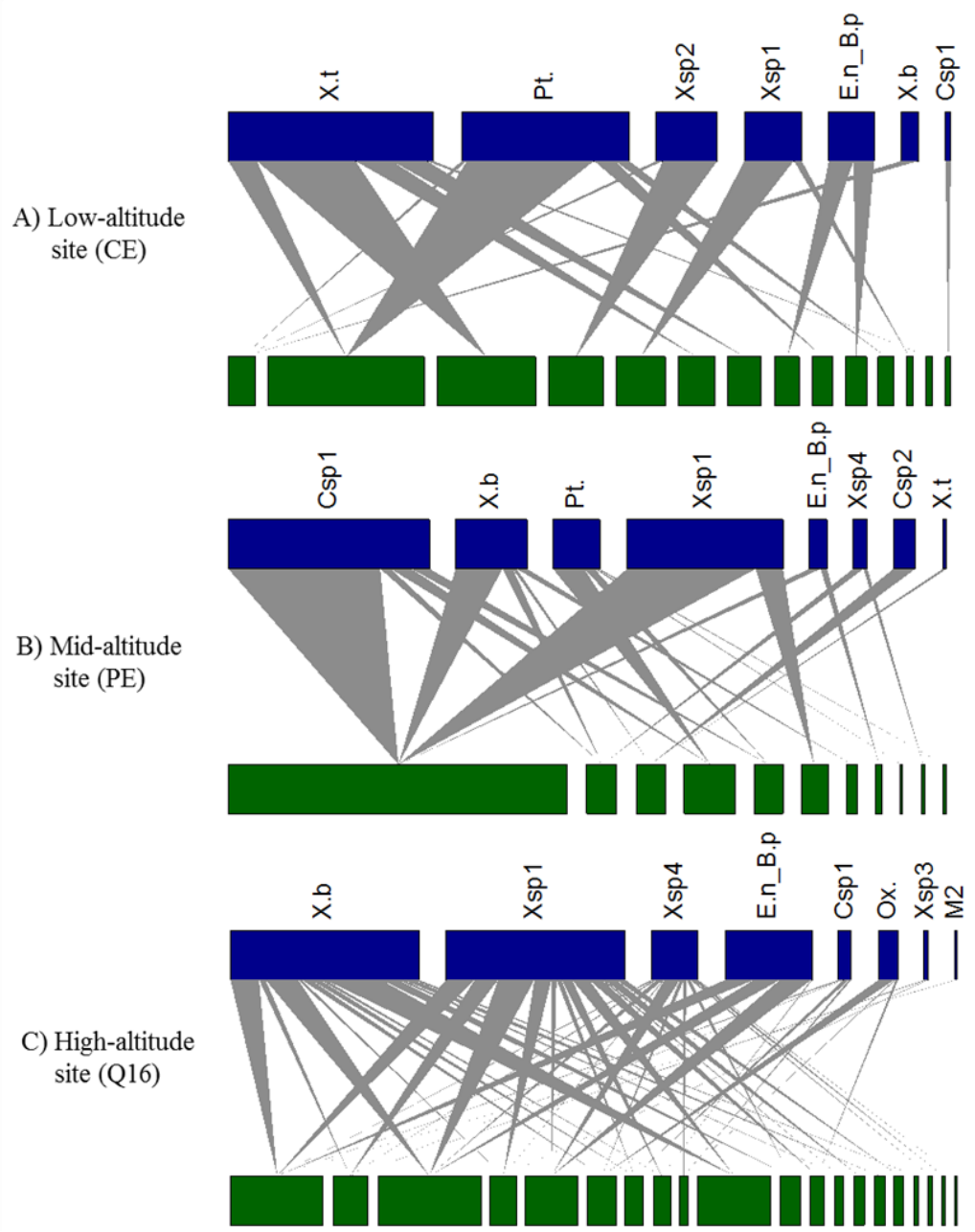

Fig.3. Individual-based plant-pollinator networks in three populations of *Trembleya laniflora* Cong. (Melastomataceae) from rocky outcrops at Serra do Cipó rupestrian grasslands, Southeastern Brazil. **(A)** CE = low-altitude site (Cedro); **(B)** PE = mid-altitude site (Pedra do Elefante), and **(C)** Q16 = high-altitude site (Quadrante 16). Green nodes represent the proportion of visited flowers by bee species for each plant individual which has interaction. Blue nodes represent the frequency of interactions of each pollinator species. Grey edges indicate the plant individual-pollinator species interactions. Csp1= *Centris* sp1, Csp2= *Centris* sp2, E.n_B.p= *Eulaema nigrita* and *Bombus pauloensis*, M2 =Morfotipo2, Ox.= *Oxaea* sp, Pt= *Ptiloglossa* sp1, X.b= *Xylocopa bimaculata*, Xsp1 = *Xylocopa* sp1, Xsp2 = *Xylocopa* sp2, Xsp3 = *Xylocopa* sp3, Xsp4 = *Xylocopa* sp4, X.t=*Xylocopa truxali*.

8. SUPPLEMENTARY INFORMATION

8.1. Tables

Table S1. Pollinator species of *Trembleya laniflora* Cong. (Melastomataceae) in three populations of *Trembleya laniflora* Cong. (Melastomataceae) from rocky outcrops at Serra do Cipó rupestrian grasslands, Southeastern Brazil. Observations conducted in July−August 2014.

Pollinator bee species
Centris sp1
Centris sp2
Bombus pauloensis Friese, 1931
Eulaema nigrita Lepeletier, 1841
Morfotipo 2
Oxaea sp
Ptiloglossa sp1 (Colletidae)
Xylocopa (Dasyxylocopa) *bimaculata* Friese, 1903
Xylocopa sp1
Xylocopa sp2
Xylocopa sp3
Xylocopa sp4
Xylocopa truxali Hurd & Moure, 1963

8.2. Figures

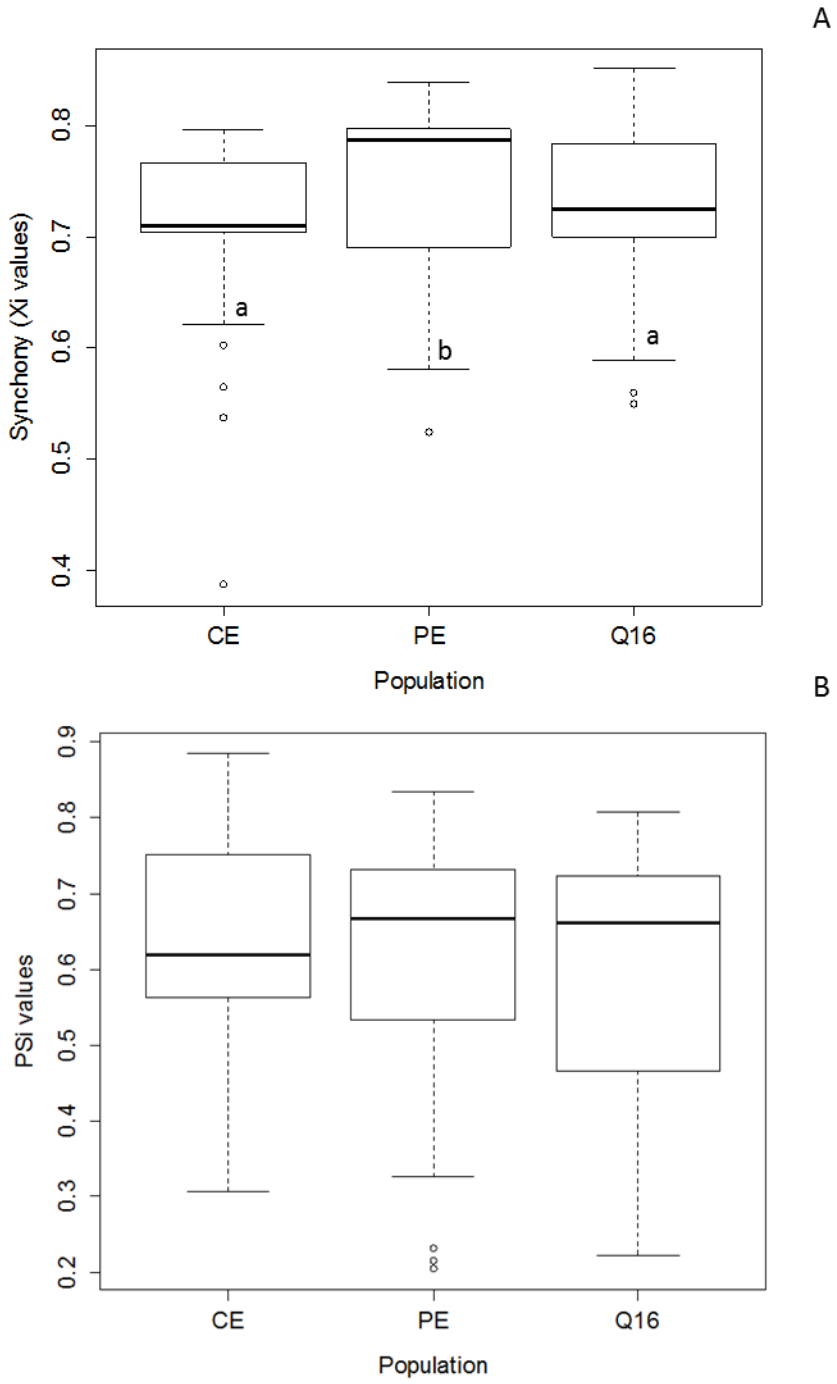

Fig.S1. Values of the **(A)** Individual synchrony (X_i) and **(B)** Proportional similarity (PS_i) indices observed in three populations of *Trembleya laniflora* (Melastomataceae) from rocky outcrops at Serra do Cipó rupestrian grasslands, Southeaster Brazil. CE = low-altitude site (Cedro); PE = mid-altitude site (Pedra do Elefante) and, Q16 = high-altitude site (Quadrante 16). Black lines represent the medians and different letters (a, b) indicate significant differences (GLM; $p<0.05$).

Appendix 1: Individual-level generalization and reproductive success in a highly specialized pollination system

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October, 2017

Contents

Introduction

In this function, we propose the estimation of relative degree of individual specialization in the use phenological niche (time) for individuals and populations of plants, contrasting the individual flowering activities with the average flowering curve of the population.
At individual-level, the function calculates the Proportional Similarity index (PSi) to each sampled individual, which measures the phenological overlap degree among the plant individuals and their population. Additionally, it calculates the Individual Specialization index (IS) at population-level (sensu Bolnick et al., 2002) and estimates the significance of observed IS considering the temporal correlation of the phenological data.
Hence, the function uses a new null model adapted to phenological data on evaluation of the IS significance. PSi and IS assume the value 1 when there is no individual specialization and lower decimal values as individual specialization becomes stronger.

Input data

Your input data is the phenological activity matrix, individuals are represented in the lines and sampling interval in the columns (bi-weekly, months, etc). The cells show the phenological activity value which can be measured as number of flowers, fruits, or intensity in Fournier classes.
Setting the working directory

```
setwd("C:/Users/VGS/Desktop/Nati")
```

Uplading files with phenological activity

```
Cedro<-read.table("C:/Users/VGS/Desktop/Nati/files_Nati/Ant_CE_actv.txt", h=T)
# Cedro
#steps to remove period without activity
# colSums(Cedro)
Cedro<-Cedro[,6:9]
# head(Cedro)

PE<-read.table("C:/Users/VGS/Desktop/Nati/files_Nati/Ant_PE_actv.txt", h=T)
# PE
# colSums(PE)
PE<-PE[,4:9]
# head(PE)

Q16<-read.table("C:/Users/VGS/Desktop/Nati/files_Nati/Ant_Q16_actv.txt", h=T)
# Q16
# colSums(Q16)
```

```r
Q16<-Q16[,5:9]
# head(Q16)
```

Code to calculate PSi and IS indices for a population

To run the function you need to set two arguments, one is your phenological matrix, called as "pop" argument, and the other is the name of your population "name" argument

```
#############################################################
##### Calculating PSi and IS for each population  ##########
#############################################################

PSi_pheno<-function(pop, name)
{
  prod_total_ind<-rowSums(pop) ## sum of all columns for each line,
  #represents the total individual production
  Pij<-as.matrix(pop/prod_total_ind) # proportional activity of each individual
  # per month regarding the total individual production
  Qj<-colSums(Pij/nrow(pop)) #Qj represent the average of phenological activity of the populatio
  Pij_mensal<-matrix(NA, nrow(pop), ncol(pop))
  for (n in 1:ncol(Pij)){
    Pij_mensal[,n]<-(abs(Pij[,n]-Qj[n]))
    }
  PSi<-as.matrix(round(1-0.5*(rowSums(Pij_mensal)), 3), nrow(pop),1)
  rownames(PSi)<-rownames(pop)
  colnames(PSi)<-c("PSi")
  PSi

  write.table(PSi, paste("C:/Users/VGS/Desktop/Nati/PSi_", name, ".txt", sep="" ))
  IS<-round(mean(PSi), 3)
  return(list("PSi"=PSi, "IS"=IS))

}
```

Running the function

```
PSi_CE<-PSi_pheno(Cedro, name="cedro")
# PSi_CE
PSi_PE<-PSi_pheno(PE, name="PE")
PSi_Q16<-PSi_pheno(Q16, name="Q16")
```

Code to calculate PSi and IS indices for simmulated populations to assess the significance of IS

Additionally, based on the null models (Monte Carlo simulations) it is possible evaluate the significance of IS values as follow:
uploading the input data (phenological matrix)

```
# Cedro<-read.table("C:/Users/VGS/Desktop/Nati/files_Nati/Ant_CE_actv.txt", h=T)
```

You need to inform two arguments to run this function. The first is the "pop" - the input data; and "aleat" - the number of simulations to run. The default is 999.

```
#######################################################
###  Testing the significance of IS  ###############

IS_signif<-function(pop, aleat=999){

IS_observed<-PSi_pheno(pop, name="")[[2]]  #to estimate the observed value of IS

#'Steps to build the simulations
indTemp <- which(pop[, ] != 0, arr.ind = T)
duration_total<-ncol(pop)  # total flowering duration for the population
matrix_duration<-matrix(NA, nrow(pop),1)

IS<-numeric(aleat)

for(m in 1:aleat){
# Creating the matrix with aleatorized start of activity
sample_temp<-matrix(NA,nrow(pop),1)
initRand <- matrix(0,nrow(pop),ncol(pop))
florRand <- matrix(0,nrow(pop),ncol(pop))

  for (i in 1:nrow(pop)) {
  # Identify all possible positions for the flowering start taking the
  # total duration into account because the physiological limits defining
  # the phenological curve need to be hold
    matrix_duration[i,1]<-duration_total-(sum(pop[i,]!=0)-1)
    # Attributing the randomized start
    sample_temp[i]<-sample(seq(1:matrix_duration[i,1]), 1)
    # Creating the simulated matrix for the start of activity
    initRand[i, sample_temp[i]]<-1
```

```
    indTemp2 <- which(pop[i, ] != 0, arr.ind = T)
      #replace the start value by the activity of each individual
    florRand[i, which(initRand[i, ]!=0)] <- pop[i, indTemp2[1, 2]]
    ifelse(nrow(indTemp2)>1,
         florRand[i, (which(initRand[i, ]!=0)+(1:(nrow(indTemp2)-1)))] <-
           as.numeric(pop[i, indTemp2[2:nrow(indTemp2), 2]]),
         florRand[i, which(initRand[i, ]!=0)] <- pop[i, indTemp2[1, 2]])
    rownames(florRand)<-rownames(pop)
    colnames(florRand)<-colnames(pop)
    ############### PSi_florRand<-PSi_pheno(florRand, name="")[[1]]
    IS_florRand<-PSi_pheno(florRand, name="")[[2]]
}

# folder to save the simulated matrices
# dir.create("C:/Users/VGS/Desktop/Nati/simu")
# write.table(florRand, paste("C:/Users/VGS/Desktop/Nati/simu/simu", m, ".txt",
# sep = ""))

IS[m]<-IS_florRand # IS for the simulated populations

}
IS_mean_simu<-mean(IS)  #average of IS values for the simulated populations
prob.E<-sum(IS>=IS_observed)  # Probability of Phenological Specialization
prob.G<-sum(IS<=IS_observed)   # Probability of Phenological Generalization

# graph to plot the results (omitted, to run just remove "#")
# hist(IS, xlim=c(0,1), col="grey", xlab=c("IS values"), main="")
# abline(v=IS_observed, col="blue")

return(list("IS randomized values"=IS,
            "Observed IS value"=IS_observed,
            "simulated mean IS"=IS_mean_simu,
            "Phenological specialization"= prob.E,
            "Phenological generalization"=prob.G))
}
```

Running the function:

```
Cedro_IS_sig<-IS_signif(Cedro, aleat=999)
Cedro_IS_sig  # results shown for one site
```

```
## $`IS randomized values`
##   [1] 0.559 0.568 0.577 0.606 0.556 0.556 0.577 0.528 0.547 0.581 0.534
##  [12] 0.571 0.552 0.597 0.569 0.531 0.539 0.568 0.556 0.539 0.568 0.558
##  [23] 0.553 0.610 0.612 0.554 0.549 0.533 0.552 0.580 0.570 0.518 0.577
##  [34] 0.557 0.571 0.567 0.524 0.566 0.540 0.545 0.502 0.535 0.579 0.557
##  [45] 0.538 0.523 0.506 0.529 0.570 0.549 0.532 0.575 0.526 0.538 0.600
##  [56] 0.600 0.565 0.538 0.567 0.561 0.560 0.553 0.588 0.556 0.528 0.545
##  [67] 0.539 0.595 0.567 0.571 0.616 0.547 0.573 0.523 0.512 0.544 0.527
##  [78] 0.607 0.555 0.560 0.534 0.536 0.544 0.572 0.524 0.549 0.564 0.534
##  [89] 0.564 0.508 0.532 0.545 0.672 0.541 0.527 0.535 0.565 0.586 0.562
## [100] 0.570 0.586 0.582 0.565 0.543 0.594 0.574 0.524 0.538 0.500 0.563
## [111] 0.533 0.542 0.583 0.539 0.554 0.654 0.584 0.544 0.573 0.546 0.546
## [122] 0.514 0.541 0.607 0.512 0.508 0.531 0.552 0.584 0.580 0.568 0.537
## [133] 0.549 0.587 0.547 0.566 0.547 0.590 0.542 0.572 0.586 0.559 0.538
## [144] 0.548 0.560 0.540 0.569 0.554 0.564 0.561 0.539 0.553 0.533 0.529
## [155] 0.571 0.569 0.574 0.612 0.585 0.583 0.556 0.603 0.536 0.566 0.548
## [166] 0.576 0.570 0.578 0.562 0.553 0.523 0.530 0.566 0.558 0.584 0.532
## [177] 0.517 0.537 0.561 0.523 0.516 0.544 0.517 0.548 0.557 0.542 0.529
## [188] 0.562 0.559 0.588 0.565 0.590 0.560 0.610 0.549 0.544 0.544 0.520
## [199] 0.590 0.564 0.524 0.548 0.566 0.552 0.566 0.546 0.559 0.574 0.618
## [210] 0.542 0.540 0.509 0.573 0.557 0.533 0.510 0.557 0.539 0.547 0.539
## [221] 0.568 0.560 0.555 0.557 0.558 0.517 0.546 0.588 0.536 0.520 0.545
## [232] 0.527 0.589 0.560 0.559 0.533 0.588 0.547 0.599 0.519 0.538 0.581
## [243] 0.571 0.645 0.533 0.552 0.554 0.579 0.545 0.545 0.541 0.548 0.538
## [254] 0.541 0.551 0.586 0.548 0.566 0.558 0.548 0.538 0.511 0.548 0.544
## [265] 0.558 0.592 0.597 0.550 0.653 0.603 0.541 0.582 0.584 0.562 0.539
## [276] 0.532 0.545 0.620 0.553 0.560 0.566 0.605 0.573 0.554 0.538 0.568
## [287] 0.538 0.556 0.536 0.543 0.540 0.562 0.569 0.550 0.540 0.562 0.524
## [298] 0.537 0.577 0.582 0.538 0.578 0.530 0.519 0.621 0.587 0.542 0.539
## [309] 0.552 0.517 0.565 0.583 0.596 0.531 0.540 0.552 0.586 0.561 0.546
## [320] 0.553 0.544 0.485 0.551 0.547 0.576 0.527 0.542 0.559 0.576 0.575
## [331] 0.540 0.524 0.574 0.540 0.535 0.513 0.548 0.573 0.520 0.602 0.536
## [342] 0.593 0.574 0.561 0.537 0.503 0.558 0.565 0.547 0.512 0.550 0.546
## [353] 0.582 0.513 0.527 0.558 0.541 0.545 0.607 0.537 0.601 0.547 0.572
## [364] 0.573 0.611 0.548 0.560 0.539 0.617 0.540 0.546 0.544 0.521 0.594
## [375] 0.517 0.533 0.602 0.552 0.575 0.541 0.588 0.544 0.546 0.548 0.546
## [386] 0.545 0.547 0.525 0.563 0.531 0.531 0.568 0.568 0.575 0.539 0.576
## [397] 0.557 0.565 0.526 0.533 0.560 0.504 0.540 0.575 0.635 0.576 0.554
## [408] 0.551 0.611 0.538 0.618 0.522 0.519 0.577 0.528 0.532 0.530 0.541
## [419] 0.560 0.568 0.583 0.551 0.509 0.541 0.552 0.544 0.534 0.544 0.544
```

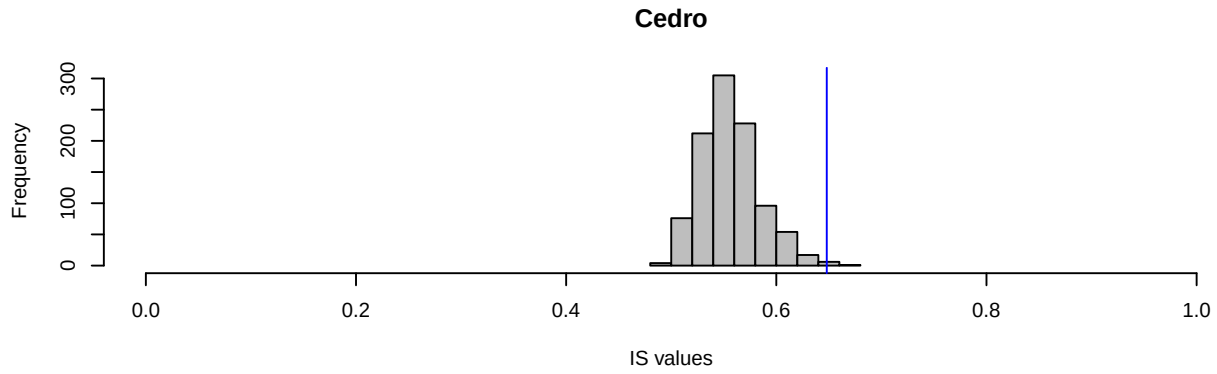
```
## [430] 0.629 0.603 0.542 0.534 0.542 0.548 0.509 0.528 0.542 0.569 0.554
## [441] 0.529 0.566 0.542 0.527 0.574 0.592 0.528 0.533 0.557 0.540 0.620
## [452] 0.560 0.607 0.557 0.565 0.552 0.537 0.554 0.548 0.549 0.617 0.542
## [463] 0.535 0.615 0.568 0.639 0.580 0.590 0.587 0.567 0.539 0.601 0.521
## [474] 0.588 0.559 0.563 0.609 0.586 0.555 0.552 0.551 0.552 0.561 0.566
## [485] 0.566 0.523 0.594 0.550 0.534 0.571 0.572 0.569 0.579 0.570 0.534
## [496] 0.503 0.568 0.559 0.547 0.527 0.519 0.525 0.583 0.542 0.573 0.537
## [507] 0.557 0.562 0.535 0.530 0.545 0.554 0.524 0.544 0.628 0.554 0.526
## [518] 0.594 0.524 0.554 0.565 0.572 0.544 0.514 0.554 0.539 0.549 0.579
## [529] 0.571 0.569 0.549 0.562 0.544 0.546 0.512 0.583 0.553 0.521 0.625
## [540] 0.518 0.544 0.567 0.541 0.558 0.520 0.565 0.551 0.547 0.548 0.525
## [551] 0.528 0.584 0.531 0.647 0.548 0.566 0.520 0.583 0.591 0.536 0.611
## [562] 0.548 0.608 0.520 0.572 0.561 0.598 0.587 0.539 0.576 0.572 0.579
## [573] 0.607 0.529 0.537 0.629 0.610 0.554 0.523 0.580 0.537 0.557 0.552
## [584] 0.575 0.570 0.547 0.568 0.513 0.505 0.592 0.572 0.574 0.593 0.545
## [595] 0.578 0.561 0.558 0.587 0.535 0.555 0.587 0.576 0.554 0.570 0.553
## [606] 0.593 0.543 0.550 0.593 0.549 0.575 0.550 0.535 0.538 0.521 0.542
## [617] 0.598 0.527 0.529 0.518 0.532 0.565 0.653 0.509 0.564 0.561 0.595
## [628] 0.524 0.552 0.555 0.608 0.561 0.566 0.552 0.568 0.565 0.576 0.588
## [639] 0.557 0.562 0.556 0.527 0.502 0.546 0.507 0.549 0.581 0.532 0.548
## [650] 0.557 0.542 0.574 0.626 0.554 0.520 0.609 0.554 0.540 0.531 0.539
## [661] 0.523 0.511 0.544 0.645 0.561 0.573 0.564 0.542 0.581 0.551 0.532
## [672] 0.542 0.561 0.546 0.511 0.563 0.487 0.560 0.540 0.565 0.612 0.561
## [683] 0.515 0.569 0.555 0.556 0.532 0.580 0.547 0.549 0.533 0.543 0.567
## [694] 0.515 0.601 0.583 0.563 0.509 0.538 0.551 0.607 0.561 0.574 0.559
## [705] 0.542 0.529 0.624 0.636 0.555 0.598 0.551 0.503 0.561 0.552 0.560
## [716] 0.529 0.567 0.533 0.596 0.514 0.566 0.565 0.520 0.612 0.592 0.584
## [727] 0.563 0.522 0.520 0.512 0.553 0.567 0.516 0.575 0.586 0.614 0.536
## [738] 0.523 0.542 0.578 0.533 0.564 0.520 0.522 0.576 0.577 0.580 0.555
## [749] 0.570 0.563 0.582 0.502 0.543 0.595 0.515 0.547 0.562 0.548 0.552
## [760] 0.523 0.571 0.534 0.569 0.540 0.573 0.570 0.569 0.501 0.544 0.544
## [771] 0.530 0.563 0.565 0.599 0.551 0.513 0.530 0.553 0.592 0.518 0.536
## [782] 0.551 0.636 0.533 0.522 0.536 0.542 0.545 0.544 0.539 0.519 0.536
## [793] 0.540 0.544 0.564 0.512 0.584 0.562 0.563 0.555 0.531 0.627 0.574
## [804] 0.555 0.539 0.545 0.538 0.605 0.556 0.528 0.538 0.535 0.559 0.562
## [815] 0.547 0.575 0.535 0.616 0.578 0.521 0.552 0.607 0.568 0.581 0.603
## [826] 0.570 0.548 0.546 0.521 0.538 0.551 0.573 0.579 0.525 0.556 0.535
## [837] 0.525 0.543 0.544 0.572 0.518 0.508 0.568 0.581 0.582 0.548 0.608
## [848] 0.604 0.604 0.579 0.522 0.542 0.533 0.585 0.552 0.514 0.527 0.621
## [859] 0.557 0.567 0.537 0.592 0.539 0.556 0.593 0.564 0.549 0.591 0.627
```

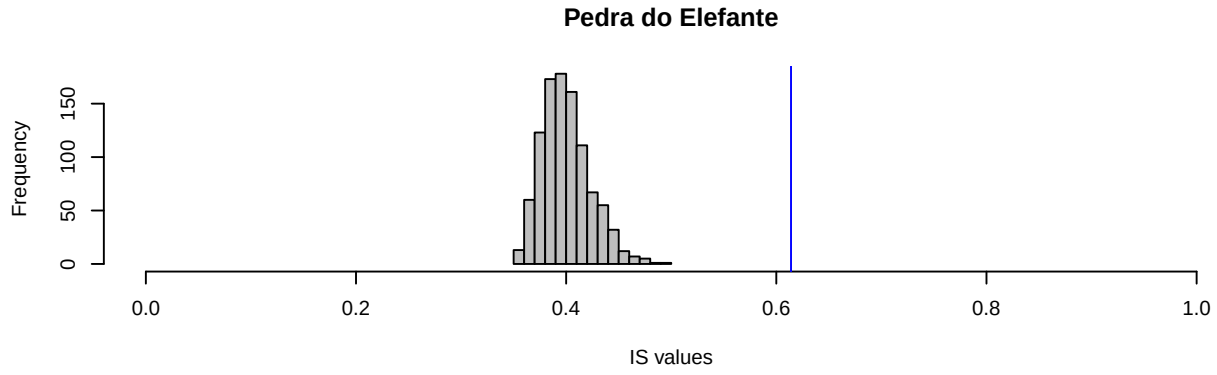
```
## [870] 0.543 0.570 0.579 0.564 0.572 0.568 0.544 0.609 0.570 0.549 0.567
## [881] 0.543 0.533 0.549 0.592 0.552 0.506 0.584 0.547 0.545 0.602 0.592
## [892] 0.519 0.548 0.532 0.528 0.551 0.561 0.537 0.569 0.565 0.558 0.557
## [903] 0.546 0.549 0.565 0.539 0.571 0.565 0.597 0.560 0.624 0.589 0.581
## [914] 0.586 0.496 0.610 0.580 0.517 0.591 0.585 0.540 0.604 0.570 0.615
## [925] 0.534 0.579 0.520 0.545 0.585 0.541 0.560 0.573 0.561 0.559 0.544
## [936] 0.549 0.562 0.551 0.533 0.521 0.525 0.541 0.539 0.537 0.541 0.571
## [947] 0.518 0.543 0.553 0.547 0.547 0.607 0.539 0.568 0.585 0.615 0.548
## [958] 0.553 0.564 0.569 0.527 0.561 0.557 0.636 0.559 0.548 0.556 0.509
## [969] 0.537 0.523 0.540 0.569 0.525 0.516 0.560 0.568 0.552 0.518 0.553
## [980] 0.546 0.543 0.558 0.534 0.507 0.528 0.630 0.574 0.572 0.573 0.537
## [991] 0.532 0.568 0.541 0.528 0.538 0.550 0.524 0.605 0.578
##
## $`Observed IS value`
## [1] 0.648
##
## $`simulated mean IS`
## [1] 0.5565475
##
## $`Phenological specialization`
## [1] 4
##
## $`Phenological generalization`
## [1] 995
```

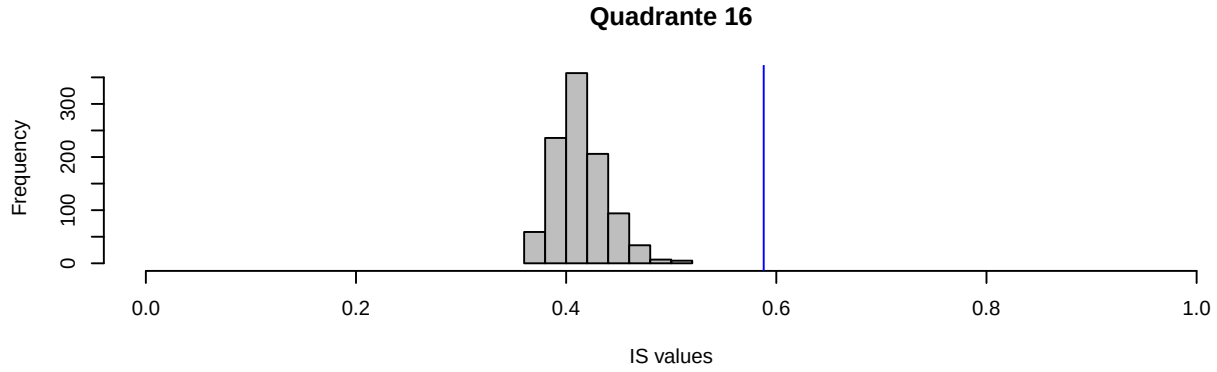
```
PE_IS_sig<-IS_signif(PE, aleat=999)
# PE_IS_sig # results ommited


Q16_IS_sig<-IS_signif(Q16, aleat=999)
# Q16_IS_sig  # results ommited

c(Cedro_IS_sig[2], PE_IS_sig[2], Q16_IS_sig[2])
```

```
## $`Observed IS value`
## [1] 0.648
##
## $`Observed IS value`
## [1] 0.614
##
## $`Observed IS value`
```

```
## [1] 0.588

#' to create a graph
 par(mfrow=c(3,1))
 hist(Cedro_IS_sig[[1]], col="grey", xlab=c("IS values"),
     xlim=c(0,1), main="Cedro")
 abline(v=Cedro_IS_sig[2], col="blue")
 hist(PE_IS_sig[[1]], col="grey", xlab=c("IS values"),
     xlim=c(0,1), main="Pedra do Elefante")
 abline(v=PE_IS_sig[2], col="blue")
 hist(Q16_IS_sig[[1]], col="grey", xlab=c("IS values"),
     xlim=c(0,1), main="Quadrante 16")
 abline(v=Q16_IS_sig[2], col="blue")
```







Saving some graphs to show the results in a jpeg file:

```
jpeg(filename=paste("C:/Users/VGS/Desktop/Nati","IS_values_all.jpg", sep="/"),
     width=14    , height=21, units = "cm", pointsize = 18, res=400,
     bg="white", restoreConsole = TRUE, type = c("windows", "cairo"))
```

```r
par(mfrow=c(3,1))
hist(Cedro_IS_sig[[1]], xlim=c(0.3,0.7), col="grey",
     xlab=c("IS values"), main="Cedro")
abline(v=Cedro_IS_sig[2], col="blue")
hist(PE_IS_sig[[1]], xlim=c(0.3,0.7), col="grey",
     xlab=c("IS values"), main="Pedra do Elefante")
abline(v=PE_IS_sig[2], col="blue")
hist(Q16_IS_sig[[1]], xlim=c(0.3,0.7), col="grey",
     xlab=c("IS values"), main="Quadrante 16")
abline(v=Q16_IS_sig[2], col="blue")
dev.off()

## pdf
##   2
```

CONCLUSÃO GERAL

Nesta tese nós apresentamos dados inéditos de biologia reprodutiva (fenologia, biologia floral, sistema reprodutivo e polinização) de *Trembleya laniflora*, uma espécie arbustiva endêmica de Melastomataceae, que apresenta características florais (flores de pólen, grandes e brancas) que destoam do padrão de flores vistosas, geralmente púrpuras, encontrado para a família, e uma distribuição restrita a afloramentos rochosos dos campos rupestres da porção sul da Cadeia do Espinhaço (Minas Gerais, Brasil). A espécie apresenta, portando, populações naturalmente isoladas no espaço. Nós demonstramos a importância das interações planta-polinizador para a polinização e sucesso reprodutivo de *T. laniflora* e descrevemos a importante relação existentente entre os distintos caracteres florais da espécie e o comportamento de forrageio de seus polinizadores principais, abelhas grandes com comportamento crepuscular. Nós observamos diferenças intraespecíficas no sucesso reprodutivo de três populações da espécie e destacamos o relevante papel das interações de polinização e da temperatura no direcionamento dessas divergências. Demonstramos também a importância de indivíduos generalistas no uso do tempo e polinizadores para o sucesso reprodutivo da espécie.

Trembleya laniflora apresenta um sistema de polinização por vibração (*buzz pollination*) altamente especializado, mediado por abelhas grandes com comportamento de forrageio crepuscular dos gêneros *Xylocopa*, *Bombus*, *Centris* e *Ptiloglossa*. Os caracteres florais distintos da espécie e sua biologia reprodutiva (antese noturna, autoincompatibilidade e grande produção de flores) foram relacionados à polinização pelas abelhas crepusculares, seus polinizadores principais e mais efetivos. Portanto, nossos resultados corroboram com a hipótese de ocorrência das "síndromes de polinização" e a existência de uma relação direta entre um conjunto de caracteres florais e o principal vetor de pólen (Faegri and Van der Pijl, 1979; Ollerton et al., 2009; Rosas-Guerrero et al., 2014). Adicionalmente, somente após os testes de germinação foi possível confirmar a autoincompatibilidade em *Trembleya*, sendo esta característica considerada como um mecanismo para evitar endogamia e favorecer a reprodução cruzada nesta espécie naturalmente isolada. Desta maneira, alertamos para a importância da realização de estudos integrativos que combinem observações de biologia floral, fenologia, sistemas reprodutivos, germinação e polinização, para o melhor conhecimento dos processos ecológicos e evolutivos que dirigem a ocorrência

das plantas nos ecossistemas naturais, e para o maior entendimento da biologia reprodutiva das espécies nativas, possibilitando ações mais efetivas para a conservação das espécies e de suas interações ecológicas.

Populações de *T. laniflora*, encontradas em áreas de diferentes altitudes e condições microambientais de temperatura, luminosidade e umidade relativa, da Serra do Cipó, diferiram em suas respostas a fatores seletivos bióticos e abióticos que, direta ou indiretamente, afetaram o sucesso reprodutivo dos indivíduos da espécie. As interações com os polinizadores atuaram como a principal pressão seletiva da fenologia de floração e biologia floral, determinando diferenças interpopulacionais na exibição das flores (sincronia, duração, produção, horário da antese e longevidade floral) que foram relacionadas a uma maior atratividade das plantas para as abelhas, os exclusivos vetores de pólen da espécie. Portanto, as interações planta-polinizador afetaram diretamente o sucesso reprodutivo de populações de *T. laniflora*. Adicionalmente, fatores abióticos, principalmente temperatura, foram relacionados a diferenças temporais na oferta dos recursos florais e dos frutos de populações de *Trembleya*, tendo influenciado as datas de início e pico das fenofases reprodutivas. Dessa maneira, confirmamos que fatores abióticos e bióticos, principalmente das interações planta-polinizador, atuam juntos como fortes pressões seletivas da biologia reprodutiva de *T. laniflora*, direcionando as variações intraespecíficas interpopulacionais nos padrões de floração e frutificação, e afetando o sucesso reprodutivo das populações, que estão espacialmente isoladas nos afloramentos de rocha dos campos rupestres do sudeste do Brasil.

Finalmente, analisando nos níveis individual e populacional as interações planta-polinizador e as atividades de floração de *Trembleya*, nós discutimos que, mesmo em sistemas altamente especializados de polinização, a generalização no uso dos recursos, tempo de floração e polinizadores, exerce papel fundamental para a conservação das interações planta-polinizador e, positivamente, afetam o sucesso reprodutivo das plantas. Nós observamos que indivíduos mais centrais e generalistas nas redes de interação planta-polinizador apresentaram maior sucesso reprodutivo quando comparados àqueles mais especialistas, e ainda, que populações compostas de indivíduos mais sincrônicos em suas respostas de floração e mais generalistas em suas interações com os polinizadores também apresentaram maior aptidão. Nós ainda apresentamos uma função para calcular os índices de especialização individual (PSi e IS, senso Bolnick et al., 2002) no uso dos recursos do nicho fenológico, e gerar modelos

nulos para testes de significância de IS, mantendo o formato das curvas fenológicas e considerando a autocorrelação temporal dos dados de fenologia. Dessa maneira, nós propomos uma maneira para testar o grau de especialização no uso do tempo fenológico e a sobreposição do nicho temporal de floração/frutificação entre indivíduos e populações.

Considerando a constante degradação, exploração e fragilidade dos campos rupestres (Silveira et al., 2016), a ocorrência restrita e endêmica de *T. laniflora* nos afloramentos rochosos, bem como, o futuro cenário de aquecimento global e sua influência no comportamento fenológico e nas interações planta-polinizador de populações e comunidades vegetais (Petanidou et al., 2014; Morellato et al., 2016), nossos resultados salientam ainda a importância da preservação e manutenção de extensas áreas de vegetação nativa, e, principalmente, dos campos rupestres, que permitam a conservação de populações espacialmente isoladas, e compostas por indivíduos que respondem diferentemente às pressões seletivas abióticas e bióticas em sua capacidade reprodutiva.

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