UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL – UFRGS

Instituto de Biociências

Programa de Pós-Graduação em Botânica

GENETIC DIVERSITY AND CONSERVATION OF *HERBERTIA ZEBRINA* DEBLE (TIGRIDIEAE: IRIDOIDEAE: IRIDACEAE), AN ENDEMIC SPECIES OF SOUTH AMERICAN GRASSLANDS

Cristiane Forgiarini

Tese submetida ao Programa de
Pós-Graduação em Botânica da UFRGS
como requerimento parcial para o
grau de Doutor em Ciências
Ênfase em Botânica.

Orientadora: Profa. Dra. Tatiana Teixeira de Souza-Chies

Coorientador: Prof. Dr. Gerhard Ernst Overbeck

Porto Alegre, Julho 2017

GENETIC DIVERSITY AND CONSERVATION OF *HERBERTIA ZEBRINA* DEBLE (TIGRIDIEAE: IRIDOIDEAE: IRIDACEAE), AN ENDEMIC SPECIES OF SOUTH AMERICAN GRASSLANDS

Cristiane Forgiarini

Instituto de Biociências

Departamento de Botânica

Universidade Federal do Rio Grande do Sul

Resumo

Frente à intensa perda de habitats naturais em decorrência do uso da terra, faz-se necessário o conhecimento prévio sobre a biologia de populações das espécies para a realização de manejo adequado e criação de estratégias de conservação. Neste sentido a avaliação de diferentes aspectos que possam auxiliar na sobrevivência da espécie e manter bons níveis de recrutamento populacional são essenciais para evitar extinções. Ao longo desta tese de doutorado uma avaliação de aspectos ecológicos e moleculares foi realizada, utilizando como modelo de estudo a espécie *Herbertia zebrina* até então considerada endêmica das pastagens naturais do Sul do Brasil.

Durante dois anos consecutivos a distribuição espacial da espécie foi estimada e populações mapeadas. Parcelas permanentes de estudos para avaliação de características fenotípicas atreladas à espécie e sua reprodução foram realizadas. A germinalibilidade de

sementes também foi avaliada e identificado que a espécie estudada necessita de um tempo maior para iniciar a germinação quando comparado a outras espécies do gênero. Os resultados gerados foram utilizados para reavaliar o *status* de conservação da espécie e as implicações destes dados na preservação da mesma foram discutidos.

Quinze loci de microssatélites (SSR) foram desenvolvidos para avaliação da diversidade genética e estrutura populacional da espécie de estudo (destes doze foram considerados polimórficos e três monomórficos). Estes marcadores moleculares também apresentaram boa transferabilidade para espécies relacionadas do gênero e por isso podem ser considerados uma ferramenta eficiente na realização de estimativas de diversidade molecular no gênero *Herbertia*.

Finalmente, utilizando os marcadores microssatélites encontrados foi obtida estimativas de diversidade genética e estrutura populacional da espécie. Os resultados sugerem que as populações de *Herbertia zebrina* estão bem estruturadas e divididas em três grupos que compartilham genes. Polinizadores foram identificados e apresentaram baixas taxas de migrantes entre populações. Estes baixos valores de migração foram atribuídos à perda de habitats e a dificuldade destes polinizadores de se deslocarem devido à fragmentação de habitats.

Esta tese de doutorado fornece novos dados sobre a biologia de populações desta espécie das pastagens naturais da América do Sul. Além disso, produziu importantes informações para o manejo das populações remanescentes da espécie estuda. A tese também serve como modelo para estudos de biologia de populações ressaltando a importância de abordagens interdisciplinares dentro de estudos de populações para uma melhor compreensão dos processos atrelados a sobrevivência de espécies.

Abstract

In times of fast losses of natural habitats and detrimental changes in land use, it is increasingly necessary to understand the population biology of endangered plant species in order to carry out proper management and adequate conservation strategies. In particular, the evaluation of key aspects of genetic diversity and population recruitment are essential to avoid extinctions. This Ph.D. thesis evaluates ecological and molecular aspects using the geophyte *Herbertia zebrina,* an endemic species from the natural grasslands of Southern Brazil.

During two consecutive years, the spatial distribution of the species was identified and population dynamics recorded. Permanent study plots were used for evaluation of reproductive traits in three representative populations. Germinability of seeds was also evaluated and it was identified that the species needs a longer time to start the germination process when compared to congeners. The results were used to reassess the conservation *status* of the species, and the implications of the results for future conservation are discussed.

Fifteen microsatellites markers (SSR) were developed to evaluate the genetic diversity and population structure of the study species (twelve were polymorphic and three monomorphic). These markers also showed good cross-amplification for related species of the genus. Therefore, these microsatellites can be considered an efficient tool in the accomplishment of estimates of molecular diversity in *Herbertia*.

Finally, using the microsatellite markers developed, the levels of genetic diversity and population structure were estimated. The results indicate that the populations are well structured and divided into three groups that sharing genes. Pollinators were identified and the low rates of migrants among populations attributed to habitat loss and the difficulties of

the pollinators to facilitate gene flow between the populations due to habitat fragmentation.

Overall, the thesis provides the first quantitative data on populations of this species of South American grasslands. In addition, it produced important information to improve management of the remaining populations of *H. zebrina*. It provides an example for population biology studies emphasizing the importance of interdisciplinary approaches in biological conservation to a better understanding of the processes linked to species survival.

Aos meus pais e irmãos, dedico.

“Go for the markers Cristiane” (Dr. Barrett)

Agradecimentos

Agradeço a professora Drª. Tatiana Teixeira de Souza-Chies por ter acreditado no meu potencial para realização deste trabalho desde o primeiro momento em que conversamos e por ter aceitado ser minha orientadora de doutorado, mesmo sabendo que meus trabalhos prévios na academia não tinham nenhuma relação com a área da biologia molecular. Agradeço a ela por ter me introduzido aos marcadores moleculares e a família Iridaceae pela qual hoje também sou completamente apaixonada. Agradeço a professora Tatiana por ter sido uma orientadora que me deixou livre para pensar e criar e quando necessário soube chamar a atenção e estar presente para me dar o suporte necessário, principalmente nos momentos mais difíceis ao longo do doutorado. Sinceramente "Tati" sou muito grata porque tu entrastes na minha vida e por ter contribuído imensamente para minha formação como pesquisadora.

Agradeço ao professor Drº. Gerhard Ernst Overbeck por ter aceitado ser meu coorientador de doutorado e ter me apoiado neste projeto interdisciplinar que até então era incomum dentro do departamento de botânica. Agradeço a ele por ter me convencido que "grama" também pode ser um objeto interessante de estudo em ecologia e que estudar estes processos pode ser tão interessante quanto o estudo de espécies arbóreas. Agradeço ao professor Gerhard por ter se tornado um amigo com o qual aprendi muito durante os trabalhos de campo e por ter sido o responsável direto pelo início do meu trabalho em colaboração com o grupo de pesquisa alemão da Universidade Técnica de Munique (TUM), Alemanha.

Agradeço ao professor Dr. Johannes Kollmann por ter me aceitado como aluna de intercâmbio durante o meu doutorado e por ter contribuído para o meu crescimento profissional. Agradeço ao professor por ter me recebido muito bem em seu país e ter me

dado suporte incondicional para realização de meu projeto de pesquisa. Agradeço a ele por ter me ajudado a melhorar minha redação científica e também pelos comentários críticos com relação ao meu trabalho que me fizeram melhorar a estrutura dos primeiros artigos desta tese de doutorado hoje já publicados e/ou aceitos. Por fim agradeço ao professor por ter acreditado imensamente no meu potencial como pesquisadora e ter me dado o privilégio de no convívio diário me ensinar lições que certamente ajudaram a melhorar meu desempenho profissional e também me tornaram uma pessoa mais forte frentes as intemperes da vida.

Agradeço ao Dr. Christian Bräuchler pelo suporto no laboratório de biologia molecular da Universidade Técnica de Munique, pelas correções na minha escrita e por ter sido o responsável pelo início do meu trabalho em conjunto com o professor Harald Meimberg da Universidade de Recursos Naturais e Ciências da Vida (BOKU) em Viena, Austria. Agradeço ao professor Harald pela disposição em incluir amostras da minha espécie de trabalho durante a preparação de duas bibliotecas de microssatélites e por ter sido o meu grande fornecedor de "*master mix*" na Alemanha para realização dos PCRs no laboratório. Agradeço também a professora Anete Pereira de Souza por ter me recebido na Universidade Estadual de Campinas (Unicamp) por um curto período de tempo, mas o suficiente para meu aprendizado em trabalhar com sequências de microssatélites e realizar os testes necessários de amplificação e genotipagem. Agradeço a Drª. Eudes Maria Stiehl-Alves e ao Dr. Manuel Curto por terem sidos os pós-doc responsáveis pelo início da minha formação no desenvolvimento de marcadores microssatélites. Por terem dividido comigo seu conhecimento na área molecular e por tere me apoiado na obtenção das sequências utilizadas ao longo deste trabalho.

Agradeço aos colegas e amigos do Laboratório de Sistemática Molecular (LABSIM), Laboratório de Taxonomia Vegetal (LabTAX), Laboratório de Estudos em Vegetação Campestre (LEVCAMP) e “agregados” da Universidade Federal do Rio Grande do Sul (UFRGS) com os quais dividi tanto tempo do meu dia na universidade que acabaram se tornando também amigos queridos. Agradeço a Anderson Luiz Christ, Alexandre Cristante Martins, Dióber Borges Lucas, Dilana Ferreira da Silva, Fernanda Schmidt Silveira, Felipe Gonzatti, Karen Araújo de Freitas, Leonardo Nogueira da Silva, Lina Maria Caballero Villalobos, Luana Olinda Tacuatiá, Marlon Facco, Suiane Santos Oleques e Tamara Pastori que me fizeram rir, mesmo nos momentos em que a vida parecia cinza. Em especial agradeço a Camila Dellanhese Inácio, Mabel Rocio Báez Lizarazo, Mariana de Souza Vieira e Thaíssa Carneiro com as quais dividi mais profundamente acontecimentos importantes da minha vida profissional e pessoal.

Agradeço também aos colegas e amigos da Universidade Técnica de Munique (TUM), Carolina Sánchez, Christina Fischer, Claudia Dieckow, Elisabeth Hamzi-Schmidt, Ferdinando Muller, Gustavo Adolfo Silva Arias, Hanna Mäerkle, Holger Paetsch, Ingrid Kapps, Jakob Huber, Janika Kerner, Julia Ditton, Katharina Strobl, Leonardo Teixeira, Marion Lang, Maximiliane Schümann e Romy Harzer. E em especial a Raquel Arias Font e Rebecca Baues pela companhia e amizade.

Aos amigos de outras caminhadas, Castor Bartolome Ruiz, Carolina Molina, Elzira Pacheco, Erika Buscardo, Inés Matilla, Jacira Dias, Julia Maria Hermann, Lairane Rekovvsky, Laura Frances, Ying Deng (vulgo “Shirley”) e Rudi e Marita Dueber que de uma forma ou outra se fizeram presentes na minha vida, e foram meus amigos mesmo apesar do tempo escasso que tive disponível fora da Universidade.

E para terminar agradeço aos meus pais e irmãos pelo apoio incondicional e pelos bons momentos em família que me proporcionam rir e descansar um pouco diante da vida agitada e cheia de “deadlines” dentro da academia. Sou muito feliz por poder contar com vocês e tê-los ao meu lado.

Este estudo foi realizado no laboratório de Sistemática Molecular (LabSIM) – Departamento de Botânica – Universidade Federal do Rio Grande do Sul, Porto Alegre/RS, Brasil. Centro de Biologia Molecular e Engenharia Genética (GBMEG) – Universidade Estadual de Campinas, Campinas/SP, Brasil. Departamento de Ecologia e Manejo de Ecossistema; Restauração Ecológica – Universidade Técnica de Munique, Weihenstephan, Freising, Alemanha. Instituto de Pesquisa Integrativa de Conservação da Natureza, Universidade de Recursos Naturais e Ciências da Vida (BOKU) Viena, Austria.

A pesquisa desenvolvida foi financiada pela Fundação O Boticário de Proteção a Natureza (Projeto 1018/20142) e pelo Conselho Nacional de Pesquisa (CNPq, Projeto nº 304197/2012-2 e 304506/2016-8). Durante o doutorado foi recebido bolsa de estudos CAPES, CAPES-PDSE e DAAD.

Sumário

Capítulo 1

Introdução geral

A extinção de espécies é normalmente dependente de fatores determinísticos relacionados à atividade humana e/ou ameaças estocásticas capazes de produzir mutações deletérias, deixando espécies vulneráveis aos efeitos de forças evolutivas (Allendorf & Luikart, 2007). De acordo com a *International Union for Conservation of Nature* (IUCN) aproximadamente 23.000 espécies no mundo estão ameaçadas de extinção (IUCN, 2015). A fragmentação de habitats tem sido citada como uma das principais causas de extinção de espécies (Pimm & Raven, 2000; Tilman et al., 2012; Godefroid et al., 2014) estando diretamente relacionada à redução da área de distribuição das populações, e por consequência, à redução do tamanho populacional das espécies (Wilson, 2016). Este processo é capaz de dividir populações em subpopulações devido à ausência de fluxo gênico e isolamento (Levin, 1970). Fragmentação de habitats representa um sério risco para espécies raras e/ou em perigo de extinção em ambientes campestres estando principalmente ligada ao uso da terra (Sala et al., 2000). Ela pode afetar o microclima e ter implicações nas taxas de sucessão ecológica (Haddad et al., 2015), ou ainda reduzir as características responsáveis pela aptidão das espécies (Waples, 2002; Charlesworth & Willis, 2009).

A redução do tamanho populacional é a maior consequência da perda de habitats e um dos três critérios usados para classificar espécies raras de acordo com a definição proposta por Rabinowitz (1981). Tamanho populacional é também um importante critério da IUCN para inclusão de espécies na lista vermelha de espécies ameaçadas (IUCN, 2017). A

redução do tamanho populacional pode aumentar os efeitos estocásticos relacionados à demografia (Melbourne, 2012) e como resultado a seleção natural pode atuar em favor de características desvantajosas para as espécies (Rabinowitz, 1981). Endogamia normalmente é uma das principais consequências da redução do tamanho populacional e normalmente associada a processos que podem levar à extinção de espécies como, por exemplo, deriva genética (Cole, 2003). Embora também possam atuar como uma barreira ao fluxo gênico interespecífico contribuindo com a especiação (Young & Clark, 2000). Seja como for, o risco de incorporar genes deletérios por causa da redução de habitats é muito maior em pequenas populações do que esperado. Alguns autores sugerem que é necessário um tamanho efetivo mínimo da população (Ne) para evitar os efeitos deletérios da endogamia no curto prazo (Franklin, 1980; Lande, 1995). Isso aumenta o risco de extinção das espécies uma vez que, em consequência da endogamia, as mesmas perdem a habilidade de se adaptar a mudanças das condições ambientais (Lande, 1995).

Estudos tem mostrado que a redução de habitats é uma das consequências diretas da fragmentação de habitats, e também está intimamente relacionada à diminuição de interações biológicas tais como a presença de polinizadores durante o período fértil (Krauss et al., 2010; Kolb, 2008). O isolamento aumenta igualmente os efeitos de aspectos microevolutivos inerentes às espécies revelando o potencial dos polinizadores no processo de adaptação natural a características florais, os quais poderão culminar em mudanças macroevolutivas (Van der Niet et al., 2014). Desde o clássico trabalho de Darwin (1876) a importância da polinização, dos atributos florais e de diferentes sistemas de reprodução para aumentar a variabilidade das espécies e evitar os efeitos da seleção natural tem sido evidenciados. Estas características são relacionadas à aptidão das espécies e tem sido

associada com níveis de heterozigosidade existentes das populações (Oostermeijer et al. 1994), bem como, utilizadas para quantificar diferenças ecológicas e variabilidade fenotípica dentro e entre espécies.

Fragmentação pode apresentar consequências negativas para a diversidade genética das espécies ao nível populacional, o que em longo prazo pode aumentar o risco de extinção de pequenas populações (Aguilar et al., 2008). Baixos níveis de diversidade genética podem não ser interessantes porque espécies com menores níveis de heterozigosidade são mais suscetíveis a forças evolutivas do que espécies com valores de heterozigosidade mais elevados (Silverstown & Charlesworth, 2001). Baixos níveis de heterozigosidade em plantas são especialmente associados com processos de endogamia (Barrett, 2014). Como citado acima, este fato pode também ser relacionados com o tipo de sistema reprodutivo das espécies, bem como, com níveis de ploidia, uma vez que, espécies diploides podem ter menores riscos de sofrerem depressão endogâmica do que espécies poliploides (Husband & Schemske, 1997), pois poliploides realizam mais frequentemente autopolinização do que espécies diploides (Mable, 2014a). Normalmente espécies que realizam polinização cruzada são consideradas mais efetivas em realizar trocas gênicas e manter altos níveis de heterozigosidade dentro e entre populações comparando a espécies autocompatíveis. Esta é a razão pela qual espécies que realizam autofertilização são conhecidas por estarem na linha final do processo evolutivo (Stebbins, 1957; Wright et al. 2013). Tanto que durante estes momentos desfavoráveis espécies que são autocompatíveis e ao mesmo tempo realizam fecundação cruzada, tendem a dar preferência pela reprodução cruzada, pois isso teoricamente pode elevar os níveis de heterozigosidade da espécie ajudando a evitar os efeitos negativos da seleção natural (Lande & Schemske, 1985). Seja como for, em situações de distúrbio, tais como fragmentação de habitats, esta situação pode ser modificada

aumentando as chances de sucesso de espécies que realizam autofertilização, pois estas não dependem de polinizadores para realização de trocas gaméticas (Lloyd, 1987).

Baixos níveis de heterozigosidade têm sido associados também à redução dos índices de plasticidade fenotípica presentes nas populações (Waples, 2002). Geralmente espécies com maior plasticidade fenotípica são consideradas responsáveis por manterem populações e tornarem espécies mais competitivas em situação adversas devido a sua ampla gama de fenótipos disponíveis (Cardoso & Lomônaco, 2003). Alguns fatores determinantes para o sucesso reprodutivo como, por exemplo, tamanho floral e tamanho da planta tem sido citados como características responsáveis por ampliar as chances das espécies de serem fecundadas (Harper, 1977; Glaettli & Barrett, 2008; Younginger, 2017). O recrutamento de novos indivíduos é considerado o último estágio para avaliar sucesso reprodutivo das espécies e a fase com os maiores índices de mortalidade em plantas (Harcombe, 1987). Tanto frutificação quanto a germinação de sementes são considerados fatores determinantes para o sucesso das espécies durante o recrutamento (Fenner & Thompson, 2000). Por este motivo, a produção de frutos e percentuais de germinação são critérios importantes para avaliar os níveis de aptidão de espécies (Solbrig et al., 1988; Rees et al., 2001). Em última instância, a avaliação de características que aumentam a aptidão das espécies está relacionada igualmente a diferentes estratégias de sobrevivência das espécies para produzirem um maior número de descendentes (Stearns, 1992). Estas características de história de vida das espécies são os fatores responsáveis pela determinação do habitat e distribuição geográfica onde as espécies são encontradas. Espécies endêmicas há muito tempo são mencionadas na literatura por apresentarem elevado nível de especialização ao ambiente onde são encontradas (Stebbins & Major, 1965) ou então por terem

características que dão certa desvantagem em relação a espécies comuns relacionadas dentro do gênero a qual pertencem (Rabinowitz, 1984).

Neste sentido, estudos populacionais que avaliem características importantes para o sucesso das espécies são essenciais para o entendimento da biologia da mesma. O primeiro artigo significativo em biologia de populações foi escrito pelo botânico Nägeli (1874), mas foi somente na década de setenta depois do trabalho de Harper (1977) que este tipo de estudo se tornou popular. Apesar do aumento do número de estudos e avaliação de características relacionadas à aptidão das espécies, estes estudos ainda são escassos. Pela falta de informações existentes, muitas espécies têm sido incluídas erroneamente em lista de espécies ameaçadas ou enquadradas em diferentes categorias que não condizem com o seu real status de ameaça (Webb 2008; Campbell, 2012). Os critérios utilizados pela IUCN para enquadrar espécies como ameaçadas exigem necessariamente à utilização de medidas de tamanho populacional e estimativas de redução de habitat para determinar se as espécies podem ser consideradas ameaçadas ou não (IUCN, 2001; 2017). Seja como for, estes parâmetros não necessariamente incluem medidas reais relacionados à aptidão e reprodução das espécies e isso tem sido alvo de crítica em estudos de biologia de populações (Collen et al., 2016; Salguero-Gómez, 2016).

Recentemente, parâmetros moleculares foram introduzidos em estudos de biologia de populações e se tornaram uma poderosa ferramenta, principalmente para avaliar a conservação de espécies. Nos dias atuais, em decorrência da alta fragmentação de habitats e perda de paisagens naturais, a avaliação de ambos os níveis, ecológico e molecular, são essenciais para obtenção de dados para o desenvolvimento de estratégias de conservação. Estas informações em conjunto são capazes de trazerem contribuições poderosas para o

correto manejo das mesmas e a escolha de áreas críticas para preservação (Silvertown & Charlesworth, 2001). Além disso, no caso de espécies consideradas endêmicas e criticamente ameaçadas de extinção como *Herbertia zebrina,* além de conservar características de aptidão das espécies, é essencial conservar a diversidade genética presente em diferentes populações para garantir a sobrevivência da mesma diante da rápida redução de habitats em que populações naturais estão sujeitas nos dias atuais.

Marcadores microssatélites

Marcadores moleculares começaram a ser utilizados em estudos de biologia de populações na década de 1970 (Allendorf & Luikart, 2007) e com o passar dos anos tornaram-se populares em estudos de diversidade genética para avaliar o nível de estruturação de populações ao longo de sua distribuição (Ouborg et al., 2010). No passado, o uso de aloenzimas foi à técnica mais popular utilizada para avaliar diversidade genética, que devido ao baixo custo ainda são correntemente utilizados em estudos populacionais (Waal et al., 2012; Anderson et al., 2016). Diferentes técnicas podem ser utilizadas para acessar a diversidade genética das espécies; como por exemplo; microssatélites (Simple Sequence Repeat (SSR)), Inter simple sequence repeat (ISSR), AFLP (Amplified fragment length polymorphism), RAPD (Restriction fragment length polymorphism) and SNPs (Single-nucleotide polymorphism) (Allendorf & Luikart, 2007). Marcadores microssatélites é um método bem estabelecido para avaliar diversidade genética das populações (Parker et al., 1998) e pode servir como base para conservação e manejo de espécies ameaçadas. Estes marcadores usam repetições *em tandem* arranjadas em 1-6 bp e apresentam herança co-dominante (Gupta et al., 1996). Estas sequências foram descritas por Litt & Luty (1989) que

detectaram sua natureza polimórfica. Comparando com RFLP, RAPD (He et al., 2003) e AFLP (Lee et al., 2004), microssatélites são considerados muito mais variáveis e informativos. Eles são considerados também mais robustos na amplificação de sequências embora muitas vezes não sejam fáceis de serem obtidos. Anteriormente o processo para encontrar microssatélites consistia na preparação de bibliotecas de DNA enriquecidas com motivos de SSR (Rassamann et al., 1991). Porém, nos últimos dez anos, o sequenciamento em larga escala, NGS (next-generation sequencing) tem facilitado o sequenciamento de alto rendimento e permitem sequenciar, por exemplo, genomas inteiros em menos de um dia (Grada & Weinbrecht, 2013). Esta tecnologia também permite identificar rapidamente um maior número de *loci* de microssatélites e desenvolver *primers* para amplificar microssatélites por um baixo custo (Senan et al., 2014). Como consequência, esta técnica tem substituído outros métodos nos últimos anos. Estudos que avaliem diversidade genética utilizando marcadores microssatélites nunca foram realizados para o gênero *Herbertia* Sweet. Embora estudos moleculares de diversidade utilizando ISSR (inter simple sequence repeat) já tenham sido realizados para o gênero (Stiehl-Alves et al., 2016; 2017).

O Sistema de estudo *Herbertia zebrina*

Herbertia zebrina Deble é membro da família cosmopolita Iridaceae que tem como centro de distribuição o continente Africano (Goldblatt et al., 1990). A família tem aproximadamente 2.030 espécies e entre 65-75 gêneros (Goldblatt et al., 2008). Iridaceae é dividida em sete subfamílias; Crocoideae, Iridoideae, Isophysidoideae, Nivenioideae, Aristeoideae, Geosiridaceae e Patersonioideae de acordo com Goldblatt e colaboradores (2008). Iridoideae é a única subfamília representada na América do Sul e é dividida em três

tribos: Tigridieae, Trimezieae e Sisyrinchieae (Goldblatt & Manning, 2008). *Herbertia zebrina* está situada dentro de Tigridieae (figura 1) que é também considerada a tribo de divergência mais recente comparando com outras tribos descritas para a América do Sul (idade estimada de 35 milhões de anos) (Golblatt et al., 2008). No Brasil a família Iridaceae é representada por 197 espécies e 23 gêneros (Flora do Brasil 2020). A alta diversidade de espécies é concentrada na região Sul do Brasil com 107 espécies descritas até agora (Flora do Brasil 2020). O alto número de representantes da família encontrado nesta região é atribuído à evolução da tribo Tigridieae juntamente com o aparecimento de recursos flores específicos denominados elaióforos. De acordo com estes estudos, o processo de especiação da tribo estaria ligado a polinizadores especializados na obtenção deste recurso floral (Chauveau et al., 2011, 2012).

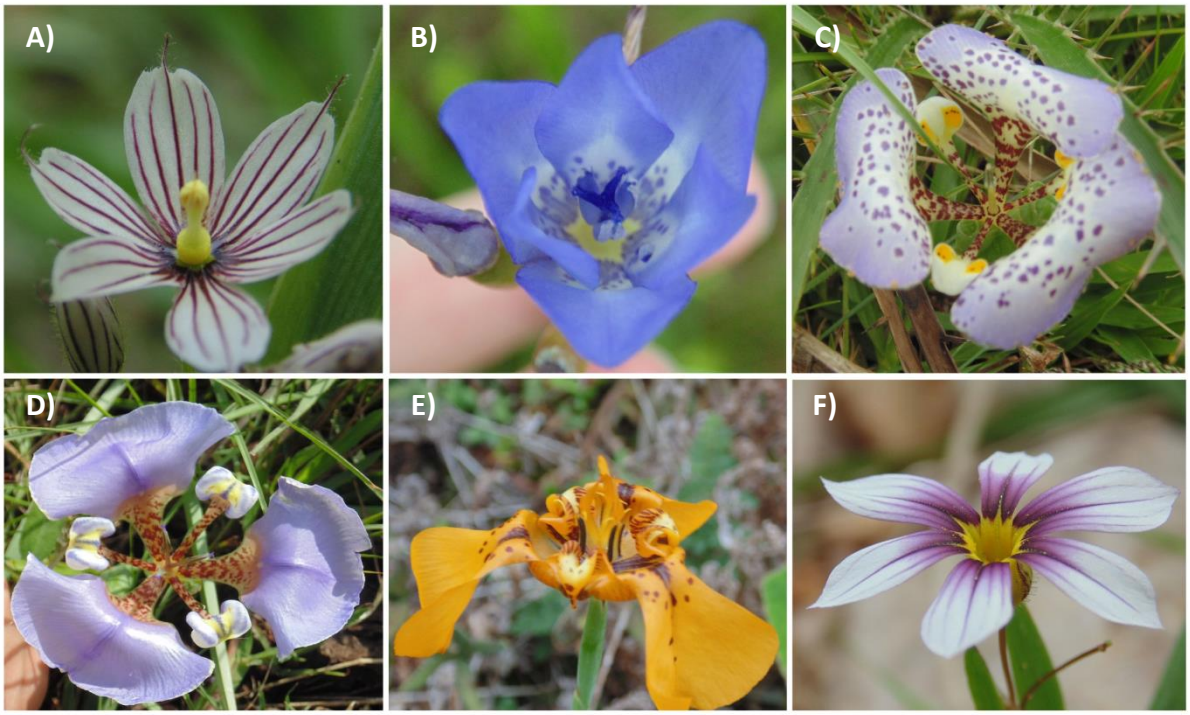

Figura 1: Representantes de Iridaceae do sul do Brasil. A) *Sisyrinchium sellowianum* Klatt, B) *Gelasine elongata* (Graham) Ravenna, C) *Kelissa brasiliensis* (Baker) Ravenna, D) *Onira unguiculata* (Baker) Ravenna, E) *Cypella herbertii* Hook, F) *Sisyrinchium micranthum* Cav.

Oito espécies são consideradas aceitas para o gênero *Herbertia* Sweet; *Herbertia pulchella* Sweet, *H. quareimana* Ravenna, *H. crosae* Roitman & J.A. Castillo, *H. darwinii* Roitman & J.A. Castillo, *H. lahue* (Molina) Goldblatt, *H. tigridioides* (Hicken) Goldblatt, *H. zebrina* e *H. amabilis* Deble. No Brasil, ocorrem sete espécies (Figura 2) sendo *H. tigridioides* a única das descritas acima que não possui registro de ocorrência no país. Por outro lado, a mais bem sucedida espécie do gênero *Herbertia* no sul do Brasil é a autocompatível *Herbertia lahue*, (Stiehl-Alves, 2016) que colonizou um grande número de países na América do Sul (Roitman & Castillo, 2004, 2008, Goldblatt & Matting, 2008) e também na América Central e do Norte (Goldblatt, 1975; Johnson, 2000). O gênero *Herbertia* é caracterizado por tricomas glandulares localizados nas tépalas internas e externas das flores, característica que aparece somente neste gênero dentro da tribo Tigridieae (Chauveau et al., 2012), pois nos demais gêneros os tricomas ocorrem somente nas tépalas internas. Diferentes níveis de ploidia têm sido relatados dentro do gênero (Stiehl-Alves, 2016) e trabalhos também tem demonstrado que espécies de *Herbertia* são capazes de armazenar em seus bulbos recursos nutricionais que estão diretamente relacionados ao processo de florescimento (Morales, 2009). Apesar disso, informações básicas dentro do gênero ainda são escassas, até mesmo dados de distribuição geográfica para algumas espécies ainda são desconhecidos.

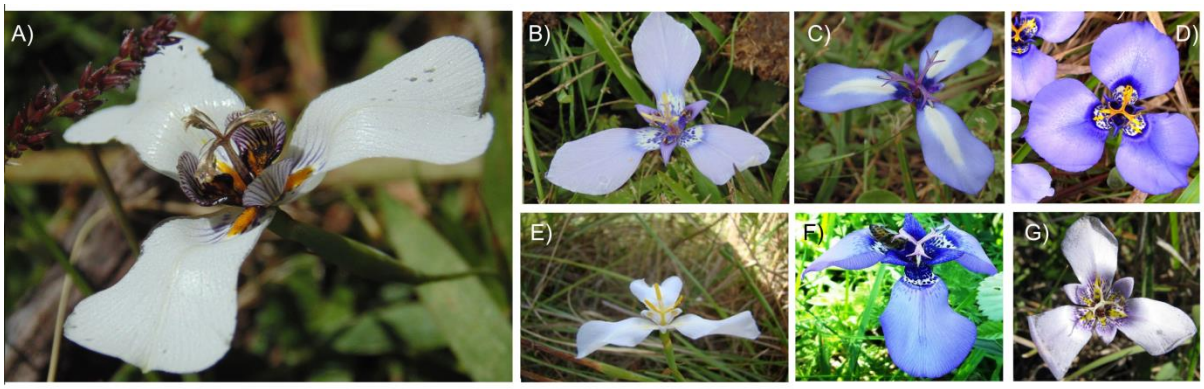

Figura 2: Espécies do gênero *Herbertia* encontradas no Brasil. (A) *Herbertia zebrina*, (B) *H. lahue*, (C) *H. pulchella*, (D) *H. darwinii*, (E) *H. amabilis*, (F) *H. quareimana* e (G) *H. crosae* (*H.*

furcata). Fotos: Cristiane Forgiarini, Eudes Maria Stiehl-Alves, Fabiano Alves e Lauís Brisolara.

Herbertia zebrina (Figura 3) é uma espécie geófita que foi descrita em 2010 como endêmica para os campos do Sul do Brasil (Deble, 2010). A espécie possui flores brancas com listas paralelas em tons de roxo escuro nas tépalas internas e externas (Deble, 2010) e elaióforos em ambas as tépalas (Chauveau et al., 2012). *H. zebrina* é uma espécie diploide (2n=14) (Moraes, 2015) perene e de reprodução cruzada (Forgiarini et al., em preparação) que foi incluída como criticamente ameaçada na lista regional de espécies do Estado do Rio Grande do Sul (Red List RS, 2014).

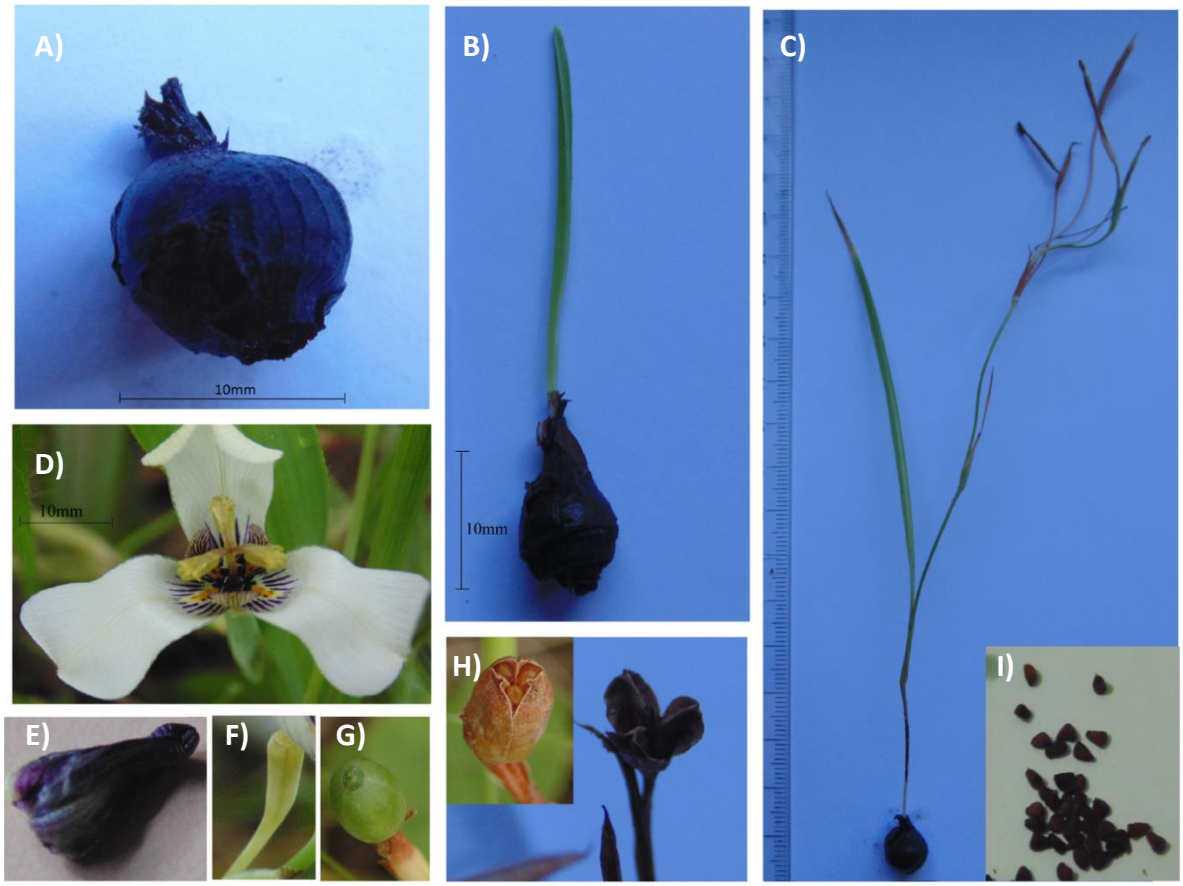

Figura 3: *Herbertia zebrina* e suas estruturas. A) Bulbo após período reprodutivo; B) Bulbo mostrando o aparecimento da primeira folha; C) Bulbo mostrando a estrutura vegetativa da espécie logo após frutificação; D) Estrutura floral; E) Estrutura floral ao final do período de abertura; F) Fruto não fecundado; G) Fruto fecundado; H) Fruto após produção de sementes e dispersão; I) Sementes.

A espécie é encontrada na extremidade norte da região conhecida como Serra do Sudeste, no Estado do Rio Grande do Sul em campos de vegetação rasa e pedregosa (Figura 4; 5a). A região de ocorrência possui formação pré-cambriana com substrato granítico e é considerada a região geologicamente mais antiga do Estado onde a espécie é distribuída (Rambo, 1956). Infelizmente, no presente momento a região vem sofrendo alta fragmentação de habitats principalmente ocasionada pelo uso da terra na silvicultura de *Pinus* sp. e *Eucalyptus* sp. (Figura 5b) (Binkowski, 2009). Devido à fragmentação, o número de pastagens naturais neste Estado desde a década de setenta vem diminuindo gradualmente e estima-se que em torno de aproximadamente 26% destes campos já tenham sido alterados (Oliveira, 2017).

Figura 4: A) Mapa de Brasil mostrando o Estado Brasileiro onde *H. zebrina* ocorre. B) Mapa identificando a região da Serra do Sudeste em cinza e a localização da área de distribuição da espécie (quadrado situado na extremidade norte da Serra do Sudeste).

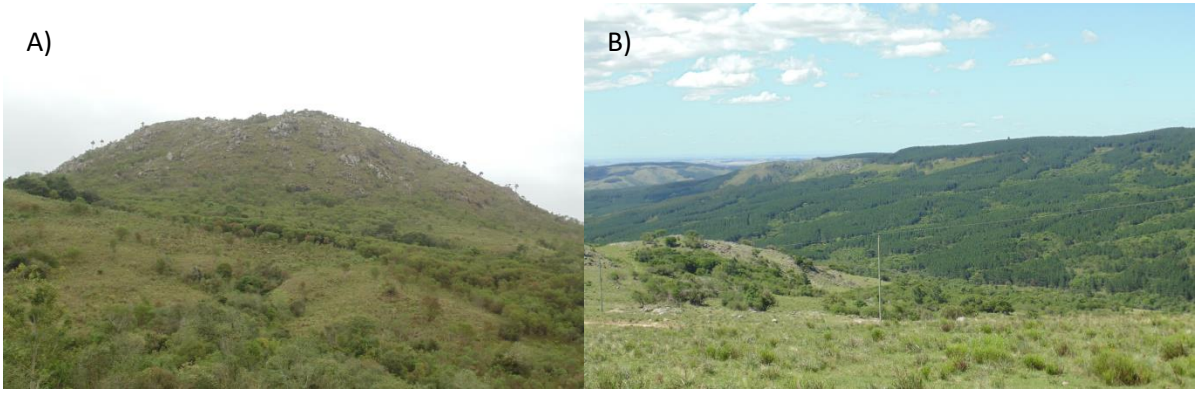

Figura 5: A) Fisionomia do local de ocorrência de *Herbertia zebrina*; B) Ao fundo da imagem, plantações de *Pinus* e a frente campo natural onde uma das populações foi localizada.

Sendo assim, se faz necessário à realização do mapeamento preciso da distribuição da espécie, bem como, o estudo populacional e a avaliação de características gerais e relacionadas os aspectos reprodutivos da mesma. Pelo mesmo motivo, estudos de diversidade genética são essenciais para traçar estratégias de manejo e conservação da espécie e com isso evitar perdas da diversidade alélica presente nas populações.

Objetivo geral da presente Tese

Estudo de aspectos ecológicos e genéticos com o intuito de contribuir para geração de informações necessárias à conservação de *Herbertia zebrina;*

Objetivos específicos da Tese

a) Estudar aspectos ecológicos de *Herbertia zebrina;*

1) Mapeamento da distribuição e tamanho populacional de *H. zebrina*;

2) Medição de características gerais e relacionadas com aptidão de *H. zebrina;*

3) Identificação do Sistema reprodutivo e polinizadores de *H. zebrina*;

b) Estudo dos aspectos genéticos de *Herbertia zebrina*

1) Desenvolvimento de marcadores microsatellites para *H. zebrina*;

2) Caracterização dos níveis de diversidade genética dentro e entre populações; estrutura e fluxo gênico.

c) Discutir sobre as implicações destes resultados para conservação da espécie.

Literatura citada

Aguilar R, Quesada M, Ashworth L, Herrerias-Diego Y, Lobo J. 2008. Genetic consequences of habitat fragmentation in plant populations: Susceptible signals in plant traits and methodological approaches. Mol. Ecol. 17: 5177–5188.

Allendorf FW and Luikart G. 2007. Conservation and the Genetics of Populations. Blackwel, Malden, Massachusetts, USA. 642p.

Anderson B, Pauw A, Cole WW, Barrett SCH. 2016. Pollination, mating and reproductive fitness in a plant population with bimodal floral-tube length. Evol. Biol. 29: 8, 1631–1642.

Barrett SCH. 2014. Evolution of Mating Systems: Outcrossing versus Selfing. (*In*) Losos, J. *The Princeton Guide to Evolution* ed. Princeton: Princeton University Press. 356-362.

Binkowski P. 2009. Conflitos ambientais e significados sociais em torno da expansão da silvicultura de eucalipto na "Metade Sul" do Rio Grande do Sul. PhD Thesis, Universidade Federal do Rio Grande do Sul, Brazil.

Campbell LM. 2012. Seeing Red: Inside the Science and Politics of the IUCN Red List.10 : 4, 367-380.

Cardoso GL, Lomônaco C. 2003. Variações fenotípicas e potencial plástico de Eugenia calycina Cambess. (Myrtaceae) em uma área de transição cerrado-vereda. Revista Brasileira de Botânica. 26:1, 131-140.

Chauveau O, Eggers L, Raquin C et al. 2011. Evolution of oil-producing trichomes in Sisyrinchium (Iridaceae): insights from the first comprehensive phylogenetic analysis of the genus. Annals of Botany 107: 1287–1312.

Chauveau O, Eggers L, Souza-Chies TT, Nadot S. 2012. Oil-producing flowers within the *Iridoideae* (Iridaceae): evolutionary trends in the flowers of the New World genera. Ann Bot 110:713–729.

Charlesworth D, and Willis JH. 2009. The genetics of inbreeding depression. Nature Reviews Genetics. 10: 783-796.

Cole CT. 2003. Genetic variation in rare and common plants. Annual Review of Ecology, Evolution and Systematics 34: 213-237.

Collen B, Dulvy NK, Gaston KJ, Gärdenfors U, Keith DA, Punt AE, Regan HM, Böhm M, Hedges S, Seddon M, Butchart SHM, Hilton-Taylor C, Hoffmann M, Bachman SP and Akçakaya HR. 2016. Clarifying misconceptions of extinction risk assessment with the IUCN Red List. Biol. Lett. 12: 20150843.

Darwin C. 1876. The effects of cross- and self-fertilization in the vegetable kingdom. John Murray, London, UK. 482p.

Deble LP. 2010. *Herbertia zebrina* (Iridaceae, Tigridieae, Cipurinae) – a new species from Rio Grande do Sul State (Brazil). Darwiniana 48: 93–96.

Fenner M and Thompson K. 2005. The Ecology of Seeds. Cambridge: Cambridge University Press. 260p.

Flora do Brasil 2020 em construção. Jardim Botânico do Rio de Janeiro. Disponível em: <http://floradobrasil.jbrj.gov.br/reflora/floradobrasil/FB127954>. Acesso em: 12 Jul. 2017.

Franklin IR. 1980. Evolutionary change in small populations. In: Soule ME, Wilcox BA eds. Conservation Biology: An Evolutionary Ecological Perspective. Sinauer Associates 135 – 140.

Godefroid S, Janssens S, Vanderborght T. 2014. Do plant reproductive traits influence species susceptibility to decline? Plant Ecol. Evol. 147: 154–164.

Goldblatt P. 1975. Revision of the bulbous Iridaceae of North America. Brittonia 27:373-385.

Goldblatt P. 1990. Phylogeny and classification of the Iridaceae. Ann Mo Bot Gard 77: 607–627.

Goldblatt P & Manning JC. 2008. The Iris Family: Natural History and Classification Timber Press. 336p.

Goldblatt P, Rodriquez A, Powell MP, Davies J, Manning JC, van der Bank M, and Savolainen V. 2008. Iridaceae 'Out of Australasia'? Phylogeny, biogeography, and divergence time based on plastid DNA sequences. Syst. Bot. 33: 495–508.

Grada A and Weinbrecht K. 2013. Next-Generation Sequencing: Methodology and Application. Research Techniques made simple. Journal of Investigative Dermatology. 133: 1-4.

Gupta PK, Balyan HS, Sharma PC, Ramesh B. 1996. Microsatellites in plants: a new class of molecular markers. Curr Sci. 70:45-54.

Haddad NM, Brudvig LA, Clobert J, Davies KF, Gonzalez A, Holt RD, Lovejoy TE, Sexton JO, Austin MP, Collins CD, Cook WM, Damschen EI, Ewers RM, Foster BL, Jenkins CN, King AJ, Laurance WF, Levey DJ, Margules CR, Melbourne BA, Nicholls A, Orrock JL, Song DX, Townshend JR. 2015. Habitat fragmentation and its lasting impact on Earth's ecosystems. Ap. Ecol.

Harcombe PA. 1987. Tree Death: Cause and Consequence. BioScience. 37, 8 : 557-568.

Harper JL. 1977. Population biology of plants. Academic Press. 892p.

He GH, Meng RH, Newman M, Gao GQ, Pittman RN, Prakash CS. 2003. Microsatellites as DNA markers in cultivated peanut (*Arachis hypogaea* L.). BMC Plant Biol 3:3.

Husband BC and Schemske DW. 1997. The effects of inbreeding in diploid and tetraploid populations of Epilobium angustifolium (Onagaraceae): implications for the genetic basis of inbreeding depression. Evol. 51:737–746.

IUCN, 2001. IUCN Red List Categories: version 3.1. Prepared by the IUCN Species Survival Commission.IUCN, Gland, Switzerland and Cambridge, UK. http://www.iucnredlist.org/static/categories_criteria_3_1. (accessed 01.07.2016).

IUCN Red List Guiding Conservation for 50 years. 2015. The IUCN Red List of threatened species. (PDF accessed 01.07.2017).

IUCN Standards and Petitions Subcommittee. 2017. Guidelines for Using the IUCN Red List Categories and Criteria. Version 13. Prepared by the Standards and Petitions Subcommittee. Downloadable from http://www.iucnredlist.org/documents/RedListGuidelines.pdf. (accessed 01.04.2017).

Johnson SR. 2000. Observations on an Autumn Flowering Population of Prairie Nymph (*Herbertia lahue* ssp. *caerulea*) in Southwestern Louisiana. The Southwestern Naturalist. 45: 2, 232-234.

Krauss J, Bommarco R, Guardiola M, Heikkinen RK, Helm A, Kuussaari M, Lindborg R, Öckinger E, Pärtel M, Pino J, Pöyry J, Raatikainen KM, Sang A, Stefanescu C, Teder T, Zobel M and Steffan-Dewenter I. 2010. Habitat fragmentation causes immediate and timedelayed biodiversity loss at different trophic levels. Ecology Letters. 13: 597–605.

Kolb A. 2008. Habitat fragmentation reduces plant fitness by disturbing pollination and modifying response to herbivory. Biol Conserv.141(10), 2540-2549.

Lande R and Schemske DW. 1985. The evolution of self-fertilization and inbreeding depression in plants. I. Genetic models. Evolution. 39:24–40.

Lande R. 1995. Mutation and conservation. Conserv biol. 9:782-91.

Lee JM, Nahm SH, Kim YM, Kim BD. 2004. Characterization and molecular genetic mapping of microsatellite loci in pepper. Theor Appl Genet. 108:619-627.

Levin R. 1970. Extinction. (*In*) Gerstenhaber, M. ed. Some Mathematical Problems in Biology. Am Math Soc. Providence, RI. pp. 77-107.

Litt M, Luty JA .1989. A hypervariable microsatellite revealed by in vitro amplification of a dinucleotide repeat within the cardiac muscle actin gene. Am J Hum Genet. 44:397-401.

Lloyd. DG 1987. Allocations to Pollen, Seeds and Pollination Mechanisms in Self-Fertilizing Plants. Funct Ecol. 1:2, 83-89.

Mable BK. 2004a. Polyploidy and self-compatibility: is there an association? New Phytologist 162: 803–811.

Melbourne BA. 2012. Demographic Stochasticity. (*In*) Hastings A and Gross LJ eds. *Encyclopedia of Theoretical Ecology. Berkeley, University of California Press. 706-712 pp.*

Moraes AP, Souza-Chies TT, Stiehl-Alves EM, et al. 2015. Evolutionary trends in Iridaceae: New cytogenetic findings from the New World. Bot J Linn Soc. 177:27–49.

Morales P, Schiappacasse F, Peñailillo P and Yañez P. 2009. Effect of bulb weight on the growth and flowering of Herbertia lahue subsp. lahue (Iridaceae). Cien Inv Agr 36:2, 259-266.

Nägeli W. 1874. Beiträge zur näheren Kenntniss der Stärkegruppe. 173: 2, 218–227.

Oliveira TE de, Freitas DS de, Gianezini M, Ruviaro CF, Zago D, Mércio TZ, Dias EA, Lampert V do N, Barcellos JOJ. 2017. Agricultural land use change in the Brazilian Pampa Biome: The reduction of natural grasslands. Land Use Policy. 63: 394–400.

Oostermeijer JGB, Van Eijck MW, den Nijs JCM. 1994. Offspring fitness in relation to population size and genetic variation in the rare perennial plant species Gentiana pneumonanthe (Gentianaceae). Oecol. 97: 289-296.

Ouborg NJ, Pertoldi C, Loeschcke V, Bijlsma R, Hedrick PW. 2010. Conservation genetics in transition to conservation genomics. Trends Genet. 26: 177-187.

Parker PG, Snow AA, Schug MD, Booton GC e Fuerst PA (1998). What molecules can tell us about populations: choosing and using a molecular marker. Ecology 79:2, 361–382.

Rabinowitz, D. 1981. Seven forms of rarity. (*In*): Synge, H. Ed. The Biological Aspects of Rare Plant Conservation. John Wiley and Sons. 205–217. 586p.

Rabinowitz D, Rapp DJ, Dixon PM. 1984. Competitive Abilities of Sparse Grass Species: Means of Persistence or Cause of Abundance. Ecology 65: 1144-1154.

Rambo B. 1956. A fisionomia do Rio Grande do Sul. Universidade do Vale do Rio dos Sinos, Brazil.

Rassmann K, Schlotterer C, Tautz D. 1991. Isolation of simple sequence loci for use in polymerase chain reactionbased DNA fingerprinting. Electrophoresis. 12:113-118.

Red List RS., 2014. Lista de espécies RS - Rio Grande do Sul. 2014. Decreto estadual n 52.109, publicado no DOE nº 233 em 02/12/2014. http://www.fzb.rs.gov.br/conteudo/4809. (accessed 01.06.2016)

Rees M. Condit R, Crawley M, Pacala S, Tilman D. 2001. Long-term studies of vegetation dynamics. Science. 293:5530, 650 – 655.

Roitman G, and Castillo A. 2004. A new species, *Herbertia crosae* (Iridaceae) from Uruguay. Brittonia 56: 361–364.

Roitman G, and Castillo A. 2008. *Herbertia darwinii* (Iridaceae: Tigridieae: Cipurinae), a new species from South America. Boletín de la Sociedad Argentina de Botánica 43: 31–314.

Sala OE, Chapin III FS, Armesto JJ, Berlow E, Bloomfield J, Dirzo R, Huber-Sanwald E, Huenneke LF, Jackson RB, Kinzig A, Leemans R, Lodge DM, Mooney HA, Oesterheld M, Poff NL, Skykes MT, Walker BH, Walker M, Wall DH. 2000. Global Biodiversity Scenarios for the Year 2100. Science 287, 1770–1774.

Salguero-Gomez R. 2016. Applications of the fast–slow continuum and reproductive strategy framework of plant life histories. New Phytologist. 213: 4, 1618–1624.

Senan S, Kizhakayil D, Sasikumar B, Sheeja TE. 2014. Methods for Development of Microsatellite Markers: An Overview. 6:1.

Silvertown & Charlesworth. 2001. Introduction to Plant Population Biology. Wiley-Blackwell. 4 ed. 360 p.

Solbrig OT, Sarandon R and Bossert W. 1988. A density-dependent growth model of a perennial herb, Viola fimbriatula. American Naturalist.

Stearns SC .1992. The evolution of life histories. Oxford University Press, New York. 248p.

Stebbins GL and Major J. 1965. Endemism and Speciation in the California Flora Ecological Monographs. 35:1, 1-35.

Stebbins GL. 1957. Self-fertilization and population variability in higher plants. Am. Nat. 91: 337–354.

Stiehl-Alves EM, Flores MSA, Heck J, Eggers L, Kaltchuk-Santos E, Mariath JEA, Souza-Chies TT. 2016. Differentiation between two self-compatible cytotypes of *Herbertia lahue* (Iridaceae): evidence from genotypic and phenotypic variation. Plant Syst Evol. 302: 6, pp 669–682.

Stiehl-Alves EM, Kaltchuk-Santos E, Eggers L, and Souza-Chies TT. 2017. Using a population approach for a preliminary investigation concerning species boundaries in *Herbertia* (Iridaceae). Int. J. Plant Sci. 178(6): 439-449.

Stuart L. Pimm and Peter Raven. 2000. Extinction by numbers. Nature. 403: 24.

Tilman D, Reich PB and Isbella F. 2012. Biodiversity impacts ecosystem productivity as much as resources, disturbance, or herbivory. Proc Natl Acad Sci U S A. 109:26, 10394–10397.

Van der Niet T, Pirie MD, Shuttleworth A, Steven D, Johnson SD, Midgley JJ. 2014. Do pollinator distributions underlie the evolution of pollination ecotypes in the Cape shrub Erica plukenetii? Part of a special issue on pollinator-driven speciation. Annals of Botany. 113: 301–315.

Younginger BS, Sirová D, Cruzan MB, and Ballhorn DJ. 2017. Is Biomass a Reliable Estimate of Plant Fitness? Applications in Plant Sciences 5(2):1600094.

Waal C, Anderson B and Barrett SCH. 2012. The natural history of pollination and mating in bird-pollinated Babiana (Iridaceae). Ann Bot. 109:3, 667–679.

Waples RS. 2002. Evaluating the effect of stage-specific survivorship on the Ne/N ratio. Mol Ecol 11:1029–1037.

Webb. GJW. 2008. The dilemma of accuracy in IUCN Red List categories, as exemplified by hawksbill turtles *Eretmochelys imbricata*. Endang Species Res. Publishing online, 24 October.

Wilson MC, Chen X-Y, Corlett RT, et al. 2016. Habitat fragmentation and biodiversity conservation: key findings and future challenges. Landsc Ecol. 31:219–227.

Wright SI, Kalisz S, and Slotte T. 2013. Evolutionary consequences of self-fertilization in plants. *Proc R Soc B*. 280:20130133.

Capítulo 2

Accepted *FLORA-D-17-00159R1*

Original Research

Using population characteristics to evaluate the conservation status of endangered grassland species – the case of *Herbertia zebrina* in southern Brazil

Cristiane Forgiarini[a,b,*], Johannes Kollmann[b], Tatiana Teixeira de Souza-Chies[a,c], Alexandre Cristante Martins[a], Eudes Maria Stiehl-Alves[a] & Gerhard Ernst Overbeck[a,c]

[a] Graduate Program in Botany, Universidade Federal do Rio Grande do Sul, Porto Alegre, Brazil

[b] Chair of Restoration Ecology, Department of Ecology & Ecosystem Management, Technical University of Munich, Germany

[c] Department of Botany, Universidade Federal do Rio Grande do Sul, Porto Alegre, Brazil

[*]Corresponding author

E-mail address: crisforgiarini@gmail.com (C. Forgiarini)

Manuscript available online *in* Flora - Morphology, Distribution, Functional Ecology of Plants (25 July 2017).

Abstract

Many plant species listed as endangered by IUCN do not have information on population characteristics and reproductive traits, although such data are needed for conservation classification. This paper revisits the conservation status of a 'critically endangered' species in fragmented grasslands of southern Brazil. We present the case of *Herbertia zebrina* (Iridaceae), a geophyte first described in grasslands on granitic outcrops in 2010. We identified 18 populations within an estimated range of about 5,000 km^2. Data on population size, plant size, fruit production and germination were recorded for three representative populations with repeated sampling of labelled flowering plants over a period of two years. Population size was 1,869–14,555 flowering plants (2.2–3.5 plants m^{-2}) with considerable turnover between years. Among populations, 11–61% new flowering individuals emerged in the second year, while 27–46% of the first-year plants were not observed in the following year. The plants emerged in early spring in both years and flowered over a period of four months. The number of fruits was positively correlated with plant size and vegetation height, while germination was about 86%. Thus, the number of populations, area of occupancy and plant reproduction indicate a more favourable conservation status than previously assumed. Nonetheless, due to population fragmentation and the rapid loss of natural ecosystems in the region because of land-use change, the overall classification of the species as 'critically endangered' should be maintained. Future research has to focus on implications of fragmentation on genetic variation among populations.

Highlights

- Abundance and reproduction of the endemic *Herbertia zebrina* are favourable.
- The species is not in an extinction vortex but endangered due to land-use change.

- The Red List classification as ‘critically endangered’ should be maintained.

Keywords: Flowering individuals, Fruit production, Geophyte, Germination, Iridaceae, Population size

1. Introduction

Worldwide, more than 8% of the plant species are currently threatened with extinction (Chapin et al., 2000). Changes in land use are among the most significant drivers of declining biodiversity (Sala et al., 2000), and habitat reduction and fragmentation have been recognized as the main direct factors of reduced population size (Wilson et al., 2016). Fragmentation can also have negative consequences on reproduction, which in the long term will increase the extinction risk of small populations (Aguilar et al., 2008). However, for many ‘threatened’ species there is a lack of data on population characteristics and reproductive traits that are needed for reliable estimates of their actual conservation status. Population studies are essential to define the Red List status of individual species. The IUCN Guidelines on Red Lists of Threatened Species (IUCN, 2001) use five criteria to determine whether a taxon is threatened or not. In general, species are evaluated based on range size, population number and size, and degree of fragmentation. Similar criteria were already suggested by Rabinowitz (1981), intended to define rarity and are still the most common measures to describe the extinction risk of species. Approximately 31% of the species included in the IUCN Red list are classified as threatened because of reduction in habitat size and increasing fragmentation (IUCN, 2017). However, for annual species with seed banks or for geophytes, estimating population size can be challenging, because of variation among seasons and years due to dormancy. Moreover, although the criteria of IUCN highlight the

importance for population viability over several generations, reproductive success is rarely evaluated for including species in Red Lists. Studies on populations of some endemic species have shown that reproduction and germination can be lower when compared with widespread species within the same family (Devoto & Medan, 2003; Brown & Botha, 2004; Carta et al., 2014). Therefore, analyses of population fluctuations and reproductive potential should be considered when classifying species as threatened or not.

The grasslands of southern Brazil include ecosystems with a particularly high number of plant species (Boldrini, 2009), many of them rare and listed as threatened (Red List RS, 2014). At the same time, the grasslands are severely affected by land-use change that can lead to ecosystem degradation (Andrade et al., 2015); protection is inefficient and conservation management poor (Overbeck et al. 2007, 2015). Moreover, the knowledge about grassland composition is sparse, hence many species are insufficiently described, and studies of their population characteristics are missing. A special feature of these grasslands is a long and asynchronous phenology for many plant species, most likely due to a short cold season and presence of different temporal niches (Oleques et al., 2017). Another challenge for understanding population dynamics might be fluctuations in plant abundance and degree of flowering over years, and more research is needed on this topic. Thus, these subtropical grasslands are complicated study systems, not only because of the emerging conservation needs, but also because the basic understanding of population dynamics of threatened species has to be improved.

Due to its diversity, the Iridaceae are a prominent family present in the grasslands in southern Brazil (Iganci et al., 2011). Many species of this family have very specific habitat requirements. They occur in small populations or have a restricted range (Munguía-Lino et al., 2016), and many are endemic (Iganci et al., 2011; Aita el al., 2013). Some Iridaceae are

negatively affected by changing land use, and populations have been markedly reduced by habitat fragmentation in past decades (Volis et al., 2010). Many Iridaceae in Brazil are considered threatened (IUCN Red List), and the systematic position of some taxa still not been well resolved (Chauveau et al., 2011; 2012; Lovo et al., 2012). Thus, there is an urgent need to improve the knowledge of the conservation status of members of this family in the grasslands of southern Brazil.

The study investigates population characteristics and reproductive traits of *Herbertia zebrina* Deble, an Iridaceae first described in 2010 (Deble, 2010), with the aiming to evaluate its conservation status." Because of its limited range size and few known populations, it is considered as 'critically endangered' (Red List RS, 2014). Specifically, it is classified as B1ab (iii,v) as a result of its small occurrence area, estimated to be less than 100 km^2, together with high fragmentation of populations and ongoing losses in population size (IUCN, 2001). Here, we investigate principal aspects of the population biology of the species and aim to re-evaluate the IUCN classification using data on distribution size, population characteristics and reproduction. Based on the results, we discuss implications for conservation measures and the challenge of close evaluations of population characteristics of threatened species in general.

2. Materials and methods

2.1. Study species and area of investigation

Herbertia zebrina Deble (Iridaceae) is a geophyte, i.e. a perennial plant whose buds ('bulbs') live below ground throughout winter, and it has a leaf height of 15–20 cm (Fig. 1). Flowers are white with dark purple parallel stripes on the external and internal tepals (Deble, 2010).

The species is included in the tribe Tigridieae and presents elaiophores on both external and internal tepals which are responsible for attracting pollinators (Chauveau et al., 2012). It is an outcrossing species pollinate by bees that seems to be affected by degradation and fragmentation of grassland habitats (Forgiarini et al., 2017; in prep.).

The study was conducted in the permanent grasslands of the most southern state of Brazil, Rio Grande do Sul, at 30°32'22.09"S and 52°42'28.77"W (Fig. 2). The geological bedrock of the region is Pre-Cambrian granite, and altitude varies from 100–400 m (Rambo, 1956). The climate is classified as Cfa according to Köppen (1936), mean annual temperatures are 17–20 °C, and precipitation 1200–1500 mm (Rossato, 2011). The economy of this region is mainly based on livestock grazing with cattle and sheep, silviculture with exotic species and, more recently, on the cultivation of grapes for wine production (IBGE, 2016).

The vegetation of the study landscape is characterized by a mosaic of low forest and grassland. *H. zebrina* occurs in grasslands with moderate densities of livestock and a rather low sward of mostly less than 5 cm height. Biodiversity of vascular grassland plants is rather high, with an average of about 35 species m^{-2}, albeit dominated by the grasses *Paspalum notanum* Flügge, *Axonopus affinis* Chase and *Piptochaetium montevidense* (Spreng.) Parodi, and the legume *Desmodium incanum* (Sw.) DC (C. Forgiarini, in prep.).

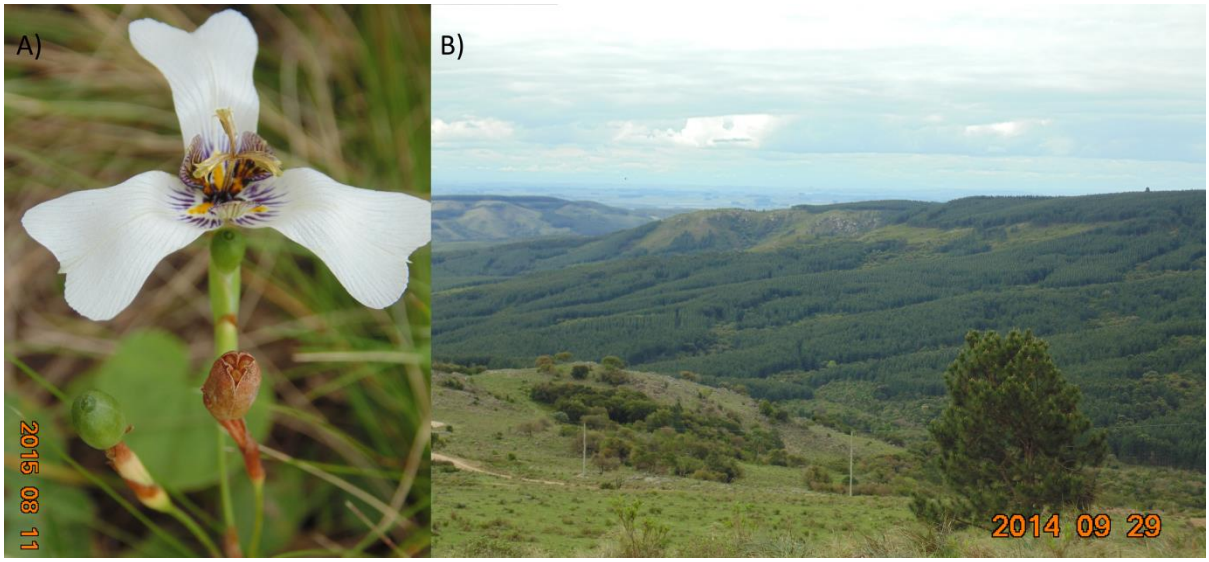

Fig. 1. (A) Flower and fruits of the endangered geophyte *Herbertia zebrina* that occurs in permanent grasslands of southern Brazil. (B) Landscape showing the grasslands covering granitic hills. Recently established pine plantations cause habitat loss and fragmentation within the endemic range of the species.

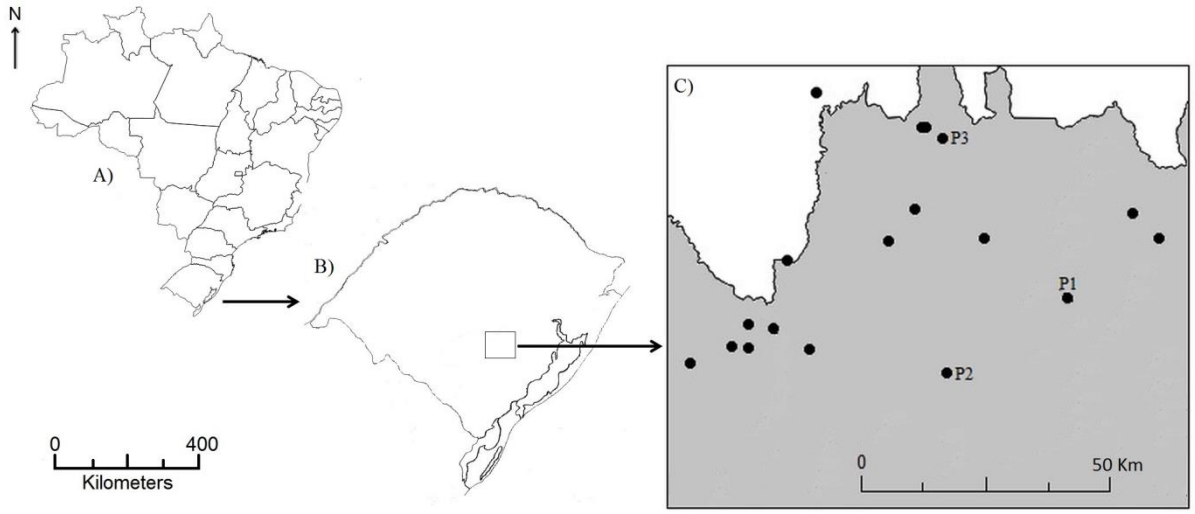

Fig. 2. Study region of the endangered study species *Herbertia zebrina*. Map of Brazil (A), the Brazilian state Rio Grande do Sul (B), and at the border between granitic highlands (grey) and lowlands (white), both naturally covered by grasslands (C). In a systematic survey, a total of 18 populations of the species were identified (black points), of which P1, P2 and P3 were used for studies of the factors controlling plant reproduction.

2.2. Measuring population characteristics and plant traits

Populations of *H. zebrina* were systematically mapped in the spring of 2013 and 2014 using QGIS 2.12.3 (QGIS, 2009). Potential study populations were identified based on satellite images of Google Earth imagery and the Geobank database (CPRM). Starting with the site for which the species has already been described, we did a survey of the entire region during the flowering period of the species (September to December).

After mapping the total area of distribution of *H. zebrina* and identification of all populations, we chose three representative ones with similar intensities of grazing for more detailed studies of population characteristics and plant reproduction (Table 1). Distances between these populations were 35–50 km. In each population, we randomly established 30 plots of 7 m x 7 m, and 30 subplots of 2 m x 2 m. The total number of flowering individuals in the populations was estimated based on the records within the 7 m x 7 m plots. Only flowering plants were recorded, since vegetative individuals were difficult to identify in the grass sward, and since species identity for non-flowering individuals was hard to determine because *Herbertia lahue* (Molina) Goldblatt and *H. pulchella* Sweet possess very similar leaves.

Within the subplots, we tagged all flowering individuals with numbered metal nails (3 cm long) fixed in the ground to analyse density, reproductive success and fitness-related variables. Care was taken not to damage the plant bulbs that are situated 4–10 cm below ground. Individuals are usually spaced by 0.1–1.5 m, so the mislabelling of individuals is unlikely. In total, 159 flowering plants (P1 = 44, P2 = 69, P3 = 46) were randomly chosen and labelled at the beginning of the flowering season in early September: in the first year 123 plants, and 36 additional plants were tagged in the second year.

The plots were revisited at least once a month from September to December in both years to cover the long flowering phenology of the species. For all individuals, we obtained data of tepal length, plant size and height of the surrounding grassland vegetation. Tepal length and plant size were measured because these variables are related with pollinator preferences, and larger plants may have higher reproductive success (Conner and Rush, 1996; Glaettli et al., 2008). Height of the surrounding vegetation was measured because this trait is associated with cattle grazing which may impact plant individuals (Gess and Gess, 1993). Grazing by cattle was not excluded, once the intention was to verify the reproductive success of the species under normal management conditions. Grazing has been common practice in the region for more than 300 years (Bell, 1998), and it is considered to be an important factor for maintenance of the grasslands and their diversity (Overbeck et al. 2007, 2016). The average number of seeds produced per plant was investigated based on a random sample of 50 fruits from the three populations (P1 = 15, P2 = 20, P3 = 15).

Table 1. Study populations of the endemic geophyte *Herbertia zebrina* in lowland grasslands of southern Brazil.

Population (name)	Coordinates	Area (ha)	Number of flowering plants	Density of flowering plants (m^{-2})
P1 (Pinheira)	S30°47.744' / W052°37.928'	1.5	1,869	3.5
P2 (PCH)	S30°40.127' / W052°23.735'	4.9	4,704	2.2
P3 (Cerro)	S30°23.857' / W052°38.416'	12.7	14,555	2.3

2.3. Germination

Germination of *H. zebrina* was analysed from March to July 2016 in a laboratory experiment, using seeds collected from 300 randomly selected individuals (P1 = 45, P2 = 75, P3 = 180).

After collection, the seeds were mixed within populations and stored for about three months at ambient temperature until the start of the experiment. Seeds were disinfected with 70% ethanol for 1 min, and washed four times with distilled water. After that, they were stored moist at 0–5 °C for two days. Then, the seeds were placed in five Petri dishes with two sheets of filter paper. Each Petri dish was divided into three sections and received 20 seeds per population. Filter papers were soaked with 3 ml of distillate water and kept humid during the experiment. Germination temperature was constant at 25 °C, with 8 h of darkness and 16 h of light, and the experiment ended when no germination was observed after a period of two weeks.

2.4. Statistical analyses

Differences among populations regarding trait values were analysed using the Kruskal-Wallis test (Zar, 1999) and with the post-hoc Dunn test in the *FSA* package (Ogle, 2016), because the data did not fit normal distributions, not even after transformation. Pearson correlation (Zar, 1999), excluding outlier values, was used to detect correlations between reproduction, plant/tepal size and grassland height. Germination of the three populations was compared using a Kaplan-Meier survivor function using the R function *survfit* from the Survival package (Therneau, 2009). All analyses were done with R version 3.3.1 (R Development Core Team, 2016).

3. Results

The comprehensive survey within the distribution range of *H. zebrina* revealed 18 populations with a total area of occupancy of ca. 1.83 km², within about 5,000 km² of

former or current grasslands (Fig. 2). Distance to the nearest population was 5–35 km. The total number of flowering plants recorded was 123 in the first year of study, and 100 in the second year. Of the 123 flowering plants tagged in the first year, 48% did not emerge in the second year, while the total proportion of new flowering individuals was 29% in the second year. The turnover of plants over years was highest in the third population (P3; 46% non-flowering plants), and less pronounced in the second population (P2).

In total, 345 fruits were recorded on the 159 individuals tagged in the field in both years: 237 fruits in the first year from the 123 individuals, and 108 fruits in the second year from 100 individuals. The fruit production per plant was 38–62% in the first year, and 20–34% in the second year (Table 2). In all areas, the percentage of fruits that did not form seeds were higher in the second year, i.e. 3.3% in P1, 50% in P2, and 42% in P3.

Table 2. Reproductive structure of the geophyte *Herbertia zebrina* in the three populations in lowland grasslands of southern Brazil.

Population (name)	P1 (Pinheira)		P2 (PCH)		P3 (Cerro)	
Year	1st	2nd	1st	2nd	1st	2nd
Total no. flowering plants	34	28	47	53	42	19
New flowering plants	0	10	0	22	0	4
Missing flowering plants	0	16	0	16	0	27
Total no. fruits	48	38	121	55	68	15
Plants with mature fruits (%)	38	34	74	24	62	20

Tepal length ($H = 9.3$, $df = 2$, $p < 0.01$) and plant height ($H = 18.5$, $df = 2$, $p < 0.001$) were higher in P2 in comparison to the other two populations, while there were no differences among sites in the mean height of the grassland sward (5.5 ± 4.3 cm, mean ± SD; Figure 3). Vegetation height was positively correlated with fruit production ($r = 0.18$, $df = 134$, $p =$

0.031), and production of fruits increased with plant size (r = 0.28, df = 155, p < 0.000), but was unrelated to tepal size (df = 138, p = 0.283).

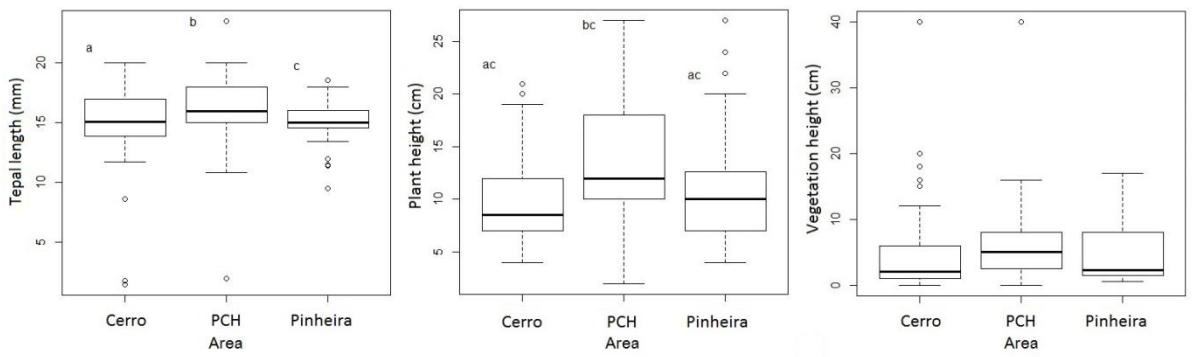

Fig. 3. Mean values of tepal length, plant size, and grassland height in three populations of the geophyte *Herbertia zebrina* studied in grasslands of southern Brazil. Different letters indicate significant differences (Dunn test, p < 0.05).

Across populations, the mean number of seeds formed per fruit was 42.7 ± 27.1 (mean ± SD). Germination was on average at 86%. In general, germination started in week 5 and was highest in weeks 7–13, while in P1 germination started two weeks later than in the other two populations and was also the last one to complete germination, approximately one month after the two others (P1 = 8–16 weeks; P2 = 5–10 weeks; P3 = 5–9 weeks). However, the difference in germination time among populations was not significant (χ^2 = 4.8, P = 0.089).

4. Discussion

4.1. Considerations about reproduction

There was considerable variation among years (and populations) in percentage of flowering plants and values of traits related to reproduction. Other studies in Iridaceae also reported

variation in fruit production among years and associated those changes with weather conditions (Szõllõsi et al., 2011). During the years of this study the variation in average temperature was approximately 1 °C for the region where *H. zebrina* is localized (INMET, 2017), which might be a driver of variation in reproduction. Another factor to be considered is that changes in temperature are also important because it affects availability of pollinators, and thus, affects reproduction (Scaven and Rafferty, 2013).

Reproduction has been reported in Iridaceae to be impaired by overgrazing (Roitman, 1998), since soil compaction and altered vegetation structure affect insect diversity and fruit set of the plants (Mayer, 2004). This may explain the positive correlation among reproduction and vegetation height, since *H. zebrina* is an outcrossing species and depends on pollinators to produce viable fruits (C. Forgiarini, unpubl. data). However, analysing a closely related species of Iridaceae (*Cypella herbertii* Hook) under different grazing regimes did not find variation in fruit set and seedling survival (Devoto and Medan, 2003). Pollination can also be the reason for the positive correlation between tepal size and reproduction, since larger flowers may attract more pollinators than small flowers (Harder et al., 2000; Worley and Barrett, 2000), but two study years are insufficient to detect such variation. This fact could be related to decreased selection of traits that give reproductive advantages for species. This parameter has also been associated with extrinsic factors such as environmental degradation to habitat fragmentation, and should be thoroughly investigated to avoid species extinction (Godefroid et al., 2014). However, considering the correlation between plant size and fruit production, it is possible that there are allometric linear relationships in *H. zebrina* (Weiner, 2004), while identifying thresholds for reproduction would need more studies (Bonser and Aarssen, 2009; Watkinson & White, 1986).

Analyses of germination in *H. zebrina* showed that the species is rather successful compared to *Herbertia lahue,* a congener widespread in South America (Schiappacasse et al., 2005). We found high germination at relatively high temperatures compared to other Iridaceae (Carta et al., 2016; Esterhuizen et al., 1986), which can be considered as adaptation to the high summer temperatures in southern Brazil. Our study also verified that germination in *H. zebrina* is rather fast and it has high yield which could indicate that the species does not have seed dormancy as described for some species of the family, especially *Crocus* spp. (Fu et al., 2013; Carta et al., 2014). Until the present moment, seed dormancy in the genus *Herbertia* were not reported in the literature, and preliminary tests with other species of the genus seem also to confirm the results found here (Martins et al., unpubl. data). On the other hand, high percentages of germination do not necessarily mean that the species has success at later life stages (Astegiano et al., 2013), because some species are poor competitors (Griggs, 1940). For more detailed information on this, plants need to be observed for longer time periods, and rates of recruitment should be verified.

4.2. Species distribution and conservation status of H. zebrina

Our study showed that *H. zebrina* has an extent of occurrence much larger than previously known (Deble, 2010). Population size was also bigger than expected: if we consider the mean number of flowering individuals estimated for the three study populations, there should be more than 100,000 flowering plants in the entire range. The current classification of the species, according to the IUCN criteria in the Red List of Rio Grande do Sul published in 2014 (B1ab[iii,v]), is based on the extent of occurrence known at the time of evaluation (one population with <100 km^2), and considering declines in size and quality of habitat,

associated with a decline in the number of mature individuals. Our data, obtained by thorough sampling in that region, indicate an extent of occurrence of up to 5,000 km^2, and an area of occupancy below 10 km^2.

While the species occurs in 18 populations, the distances between them maybe would be considered too large to allow frequent gene flow, which may suggest classification as 'severely fragmented' (IUCN, 2017), and thus, the acceptance of criterion B2a. Considering that the region of occurrence is under rapid land-use change, mostly due to silviculture expansion (Binkowski, 2009; Oliveira et al. 2017), in combination with low protection levels (Overbeck et al., 2015), it is justified to project declines in the area of occupancy and habitat, thus accepting criterion B2b(ii,iii). Thus, our overall classification of the species, according to the IUCN criteria ('critically endangered'), does not change, while the new population data are more robust and detailed.

Our results allow for clear considerations of the conservation status of *Herbertia zebrina*, and also show the importance of population biology studies for this kind of evaluation. While the data are insufficient for calculating population viability analyses (as stipulated by IUCN criteria A), the information on germination and population-level fluctuations indicate no reproductive bottleneck. We did not study plant establishment in the field, but our results on population size indicate that there should at least be no severe problem, unless land management had changed very recently. Matrix modelling of population dynamics could be used to make more detailed predictions about population dynamics, but information on age, structure and turnover of the populations would then be required (Morris and Doak, 2002). Long-term studies are necessary to evaluate population dynamics (e.g. Sarukhán and Harper, 1973). At the same time, plants have different life histories and

strategies (Stearns, 1992), and can have distinct responses at different interactions with intrinsic and extrinsic factors (Pärtel et al., 2005; Flather and Sieg, 2007).

Nevertheless, our data suggest that the species has a degree of reproduction that is much higher than in other species that are considered threatened (Devoto and Medan, 2003), which is good news concerning *H. zebrina*. Nonetheless, as long as habitat reduction and fragmentation by rapid land-use change in the region are not reduced, the species will remain under high threat (Binkowski, 2009), thus, justifying the inclusion of *H. zebrina* in the Red List. In addition to the existing knowledge, it seems important to evaluate potential effects of population fragmentation on genetic variation within and amongst populations (Silvertown et al., 2005).

The southern Brazilian grasslands are exposed to several threats, principally by the establishment of monocultures of crops such as soybean, rice, *Pinus* sp., *Eucalyptus* sp. and *Acacia* sp. in native grasslands (Oliveira, 2017). This ongoing habitat fragmentation is a serious and direct risk for endemic species such as *H. zebrina* that occur in this region. Populations can get lost due to rapid land-use change, and hence, remaining populations will become fragmented. Nonetheless, the distribution area of *H. zebrina* is considerably larger than what was known before, and the number of individuals within populations is higher than expected. Moreover, fruit production is positively associated with an increase in vegetation height. For the South Brazilian grasslands, it is well established that abandonment leads to dominance of tall grasses and shrub encroachment (Overbeck et al., 2005, Lezama et al., 2013), which has been shown to result in marked changes in species composition and vegetation structure, in general (Koch et al., 2016). Thus, total exclusion of cattle would not be a good strategy for conservation of *H. zebrina*, even more considering that it is a species with low stature that likely would suffer from out-shading and littler

accumulation. On the other hand, there might be a limit for grazing intensity after which it becomes detrimental for the species. More detailed studies on this topic are necessary to establish more precise guidelines about grazing management.

5. Conclusions

The single information about *H. zebrina* prior to our study was its taxonomic description and a Red List evaluation based on the only known population. During our study, populations were mapped, tagged individuals followed, and fruit production and germination were investigated. The results confirm that *H. zebrina* is a threatened endemic species, according to IUCN guidelines (critically endangered B2ab(ii,iii)), now based on more robust data. Our data also give directions for conservation programs of the species under current and future land use. Looking beyond the plant species, the results illustrate the importance of research on the population dynamics and reproduction of threatened species in South American grasslands. More generally, reliable data concerning fruit production and germination are necessary to include species in Red Lists. Furthermore, this information together with data on range size, population numbers, and degree of fragmentation are essential for designing future conservation programs.

Acknowledgements

We thank Geraldo Luiz Gonçalves Soares for support in the germination experiments, Julia-Maria Hermann for suggestions on earlier version of the manuscript, and Elizabeth Hamzi-Schmidt for linguistic corrections. This work was supported by *Fundação Grupo Boticário de Proteção a Natureza* [grant number: 1018/20142]. Cristiane Forgiarini and Eudes Maria

Stiehl Alves thank to Capes for the grants. Gerhard Ernst Overbeck and Tatiana Teixeira de Souza-Chies acknowledge CNPq [grant numbers: 310022/2015-0, 304197/2012-2 and 304506/2016-8]. Johannes Kollmann was supported by the German Research Foundation [grant number: KO1741/3-1].

References

Aguilar, R., Quesada, M., Ashworth, L., Herrerias-Diego, Y., Lobo, J., 2008. Genetic consequences of habitat fragmentation in plant populations: Susceptible signals in plant traits and methodological approaches. Mol. Ecol. 17, 5177–5188. doi:10.1111/j.1365-294X.2008.03971.x.

Aita, A.M., Chauveau, O., Souza-Chies, T.T., Eggers, L., 2013. Two new species of *Sisyrinchium* (Iridaceae) from subtropical highland grasslands of Southern Brazil. Phytotaxa 88, 9–18. doi:10.11646/phytotaxa.88.1.3.

Andrade, B.O., Koch, C., Boldrini, I.I., Vélez-Martin, E., Hasenack, H., Hermann, J.M., Kollmann, J., Pillar, V.D., Overbeck, G.E., 2015. Grassland degradation and restoration: A conceptual framework of stages and thresholds illustrated by southern Brazilian grasslands. Braz. J. Nat. Conserv. 13, 95–104. doi:10.1016/j.ncon.2015.08.002.

Astegiano, J., Funes, G., Galetto, L., 2013. Plant recruitment and range size: Transition probabilities from ovule to adult in two *Ipomoea*. Acta Oecol. 48, 76–82. doi:10.1016/j.actao.2013.02.005.

Bell, S., 1998. Campanha Gaúcha. A Brazilian Ranching System, 1850–1920. Stanford University Press, California.

Binkowski, P., 2009. Conflitos ambientais e significados sociais em torno da expansão da silvicultura de eucalipto na “Metade Sul” do Rio Grande do Sul. PhD thesis, Universidade Federal do Rio Grande do Sul, Brazil.

Boldrini, I.I., 2009. A flora dos campos do Rio Grande do Sul. In: Pillar, V.D., Müller, S.C., Castilhos, Z.M.S., Jacques, A.V.A. (Eds.), Campos Sulinos: Conservação e Uso Sustentável da Biodiversidade. Ministério do Meio Ambiente. Brasília, pp. 63–77.

Bonser, S.P., Aarssen, L.W., 2009. Interpreting reproductive allometry: Individual strategies of allocation explain size-dependent reproduction in plant populations. Perspect. Plant Ecol. Evol. Syst. 11, 31–40. doi:10.1016/j.ppees.2008.10.003.

Brown, N.A.C., Botha, P.A., 2004. Smoke seed germination studies and a guide to seed propagation of plants from the major families of the Cape Floristic Region, South Africa. S. Afr. J. Bot. 70, 559–581. doi:10.1016/S0254-6299(15)30194-0.

Carta, A., Hanson, S., Müller, J. V., 2016. Plant regeneration from seeds responds to phylogenetic relatedness and local adaptation in Mediterranean *Romulea* (Iridaceae) species. Ecol. Evol. 6, 4166–4178. doi:10.1002/ece3.2150.

Carta, A., Probert, R., Moretti, M., Peruzzi, L., Bedini, G., 2014. Seed dormancy and germination in three *Crocus* ser. *Verni* species (Iridaceae): Implications for evolution of dormancy within the genus. Plant Biol. 16, 1065–1074. doi:10.1111/plb.12168.

Chapin, F.S., Zavaleta, E.S., Eviner, V.T., Naylor, R.L., Vitousek, P.M., Reynolds, H.L., Hooper, D.U., Lavorel, S., Sala, O.E., Hobbie, S.E., Mack, M.C., Díaz, S., 2000. Consequences of changing biodiversity. Nature 405, 234–242. doi:10.1038/35012241.

Chauveau, O., Eggers, L., Raquin, C., Silvério, A., Brown, S., Couloux, A., Cruaud, C., Kaltchuk-Santos, E., Yockteng, R., Souza-Chies, T.T., Nadot, S., 2011. Evolution of oil-producing trichomes in *Sisyrinchium* (Iridaceae): Insights from the first comprehensive phylogenetic analysis of the genus. Ann. Bot. 107, 1287–1312. doi:10.1093/aob/mcr080.

Chauveau, O., Eggers, L., Souza-Chies, T.T., Nadot, S., 2012. Oil-producing flowers within the Iridoideae (Iridaceae): evolutionary trends in the flowers of the New World genera. Ann. Bot. 110, 713–729. doi:10.1093/aob/mcs134.

Conner, J.K., Rush, S., 1996. Effects of flower size and number on pollinator visitation to wild radish, *Raphanus raphanistrum*. Oecol. 105, 509 –516. doi:10.1007/BF00330014.

Deble, L.P., 2010. *Herbertia zebrina* (Iridaceae, Tigridieae, Cipurinae) – a new species from Rio Grande do Sul State (Brazil). Darwiniana 48, 93–96.

Devoto, M., Medan, D., 2003. Effects of grazing disturbance on the reproduction of a perennial herb, *Cypella herbertii* (Lindl.) Herb. (Iridaceae). Plant Syst. Evol. 243, 165–173. doi:10.1007/s00606-003-0084-0.

Esterhuizen, A.D., Van de Venter, H.A., Robbertse, P.J., 1986. A preliminary study of seed germination of *Watsonia fourcadei*. S. Afr. J. Bot. 52, 221–225. doi:10.1016/S0254-6299(16)31553-8.

Flather, C.H., Sieg, C.H., 2007. Species rarity: definition, causes, and classification. In: Raphael, M.G., Molina, R. (Eds.), Conservation of rare or little-known species: biological, social, and economic considerations. Island Press, pp. 40–66.

Fu, Z., Tan, D., Baskin, J.M., Baskin, C.C., 2013. Seed dormancy and germination of the subalpine geophyte *Crocus alatavicus* (Iridaceae). Aust. J. Bot. 61, 376. doi:10.1071/BT13098.

Gess, F.W., Gess, S.K., 1993. Effects of increasing land utilization on species representation and diversity of aculeate wasps and bees in the semiarid areas of Southern Africa. In:

LaSalle J., Gauld, L.D., (eds.) Hymenoptera and biodiversity. Wallingford: CAB International, pp. 83–113.

Godefroid, S., Janssens, S., Vanderborght, T., 2014. Do plant reproductive traits influence species susceptibility to decline? Plant Ecol. Evol. 147, 154–164. doi:10.5091/plecevo.2014.863.

Glaettli, M., Spencer, C., Barrett, H., 2008. Pollinator responses to variation in floral display and flower size in dioecious *Saqittaria latifolia* (Alismataceae). New Phytol. 179, 1193–1201. doi: 10.1111/j.1469-8137.2008.02532.x.

Griggs, R.F., 1940. The ecology of rare plants. Bull. Torrey Bot. Club. 67, 575–594. http://www.jstor.org/stable/2481578.

Harder, L.D., Barrett, S.C.H., Cole, W.W., 2000. The mating consequences of sexual segregation within inflorescences of flowering plants. Proc. R. Soc. London B Biol. Sci. 267, 315–320. doi:10.1098/rspb.2000.1002.

IBGE, 2017. Instituto Brasileiro de Geografia e Estatística. Cidades. Rio Grande do Sul. Encruzilhada do Sul. Histórico. http://cidades.ibge.gov.br/xtras/perfil.php?codmun=430690/ (accessed 01.02.17).

Iganci, J.R. V, Heiden, G., Miotto, S.T.S., Pennington, R.T., 2011. Campos de Cima da Serra: The Brazilian subtropical highland grasslands show an unexpected level of plant endemism. Bot. J. Linn. Soc. 167, 378–393. doi:10.1111/j.1095-8339.2011.01182.x.

INMET, 2017. Instituto Nacional de Meteorologia. Estações e dados. Dados em gráficos. Estações Convencionais. http://www.inmet.gov.br/portal/index.php?r=home/ (accessed 15.012.2016).

IUCN, 2001. IUCN Red List Categories: version 3.1. Prepared by the IUCN Species Survival Commission.IUCN, Gland, Switzerland and Cambridge, UK. http://www.iucnredlist.org/static/categories_criteria_3_1. (accessed 01.07.2016).

IUCN Standards and Petitions Subcommittee. 2017. Guidelines for Using the IUCN Red List Categories and Criteria. Version 13. Prepared by the Standards and Petitions Subcommittee. Downloadable from http://www.iucnredlist.org/documents/RedListGuidelines.pdf. (accessed 01.04.2017).

Koch, C., Conradi, T., Gossner, M.M., Hermann, J.M., Leidinger, J., Meyer, S.T., Overbeck, G.E., Weisser, W.W., Kollmann, J., 2016. Effects of management intensity and temporary conversion to other land use on plant diversity and species composition of subtropical grasslands in southern Brazil. Appl. Veg. Sci. 19, 553–749.

Köppen, W., 1936. Das geographische system der klimate. Handb. der Klimatologie 7–30. doi:10.3354/cr01204.

Lezama, F., Baeza, S., Altesor, A., Cesa, A., Chaneton, E.J., Paruelo, J.M., 2014. Variation of grazing-induced vegetation changes across a large-scale productivity gradient. J. Veg. Sci. 25, 8–21. doi:10.1111/jvs.12053.

Lovo, J., Winkworth, R.C., Mello-Silva, R., 2012. New insights into *Trimezieae* (Iridaceae) phylogeny: what do molecular data tell us? Ann. Bot. 110, 689–702. doi:10.1093/aob/mcs127.

Mayer, C., 2004. Pollination services under different grazing intensities. Int. J. Trop. Insect Sci. 24, 95–103. doi:10.1079/IJT20047.

Morris, W. F., and D. F. Doak. 2002. Quantitative conservation biology: Theory and practice of population viability analysis. Sinauer Associates, Sunderland, USA.

Munguía-Lino, G., Escalante, T., Morrone, J.J., Rodríguez, A., 2016. Areas of endemism of the North American species of Tigridieae (Iridaceae). Aust. Syst. Bot. 29, 142–156. doi:10.1071/SB16002.

Ogle, D.H., 2016. FSA: Fisheries Stock Analysis. R package version.0.8.9. Vienna: R Foundation for Statistical Computing: Vienna. https://cran.r-project.org/web/packages/FSA/FSA.pdf. (accessed 05.06.2016).

Oleques, S.S., Overbeck, G.E., de Avila, R.S., 2017. Flowering phenology and plant-pollinator interactions in a grassland community of Southern Brazil. Flora 229, 141–146. doi:10.1016/j.flora.2017.02.024.

Oliveira, T.E. de, Freitas, D.S. de, Gianezini, M., Ruviaro, C.F., Zago, D., Mércio, T.Z., Dias, E.A., Lampert, V. do N., Barcellos, J.O.J., 2017. Agricultural land use change in the Brazilian Pampa Biome: The reduction of natural grasslands. Land Use Policy 63, 394–400. doi:10.1016/j.landusepol.2017.02.010.

Overbeck, G.E., Müller, S.C., Fidelis, A., Pfadenhauer, J., Pillar, V.D., Blanco, C.C., Boldrini, I.I., Both, R., Forneck, E.D., 2007. Brazil's neglected biome: The South Brazilian Campos. Perspect. Plant Ecol. Evol. Syst. 9, 101–116. doi:10.1016/j.ppees.2007.07.005.

Overbeck, G.E., Müller, S.C., Pillar, V.D., Pfadenhauer, J., 2005. Fine-scale post-fire dynamics in southern Brazilian subtropical grassland. J. Veg. Sci. 16, 655–664. doi:10.1111/j.1654-1103.2005.tb02408.x.

Overbeck, G.E., Vélez-Martin, E., Scarano, F.R., Lewinsohn, T.M., Fonseca, C.R., Meyer, S.T., Müller, S.C., Ceotto, P., Dadalt, L., Durigan, G., Ganade, G., Gossner, M.M., Guadagnin, D.L., Lorenzen, K., Jacobi, C.M., Weisser, W.W., Pillar, V.D., 2015. Conservation in Brazil needs to include non-forest ecosystems. Divers. Distrib. 21, 1455–1460. doi:10.1111/ddi.12380.

Overbeck, G.E., Ferreira, P.M.A., Pillar, V., 2016. Conservation of mosaics calls for a perspective that considers all types of mosaic-patches. Reply to Luza et al. Braz. J. Nat. Conserv. 14, 152–154. http://dx.doi.org/10.1016/j.ncon.2016.05.002.

Pärtel, M., Kalamees, R., Reier, Ü., Tuvi, E.L., Roosaluste, E., Vellak, A., Zobel, M., 2005. Grouping and prioritization of vascular plant species for conservation: Combining natural rarity and management need. Biol. Conserv. 123, 271–278. doi:10.1016/j.biocon.2004.11.014.

QGIS 2.12.3., 2009. QGIS Geographic Information System. Organization Open Source Geospatial Foundation. http://qgis.osgeo.org. (accessed 01.03.2016).

Rabinowitz, D., 1981. Seven forms of rarity. In: Synge, H. (Ed.), The Biological aspects of rare plant conservation. John Wiley and Sons, pp. 205–217. doi:10.1177/004728702237415.

Rambo, B., 1956. A fisionomia do Rio Grande do Sul. Universidade do Vale do Rio dos Sinos, Brazil.

R Development Core Team., 2016. R: A language and environment for statistical computing. R Foundation for Statistical Computing. Version 3.3.1. Vienna, Austria. https://www.R-project.org. (accessed 01.06.2016).

Red List RS., 2014. Lista de espécies RS – Rio Grande do Sul. 2014. Decreto estadual n 52.109, publicado no DOE nº 233 em 02/12/2014. http://www.fzb.rs.gov.br/conteudo/4809. (accessed 01.06.2016)

Roitman, G.G., 1998. Efecto de los distúrbios sobre el sistema reproductivo de las Angiospermas: las comunidades vegetales de los pastizales de la Depresión del Salado, provincia de Buenos Aires. Magister scientiae thesis. University of Buenos Aires. Argentina.

Rossato, M.S., 2011. Os climas do Rio Grande do Sul: Variabilidade, tendências e tipologia. PhD thesis, Universidade Federal do Rio Grande do Sul, Brazil.

Sala, O.E., Chapin III, F.S., Armesto, J.J., Berlow, E., Bloomfield, J., Dirzo, R., Huber-Sanwald, E., Huenneke, L.F., Jackson, R.B., Kinzig, A., Leemans, R., Lodge, D.M., Mooney, H.A., Oesterheld, M., Poff, N.L., Skykes, M.T., Walker, B.H., Walker, M., Wall, D.H., 2000. Global biodiversity scenarios for the year 2100. Science 287, 1770–1774. doi:10.1126/science.287.5459.1770.

Sarukhán, J., Harper, J.L., 1973. Studies on plant demography: *Ranunculus repens* L., *R. bulbosus* L. and *R. acris* L.: I. Population flux and survivorship. J. Ecol. 61, 675–716. doi:10.2307/2258643.

Scaven, V.L., Rafferty, N.E., 2013. Physiological effects of climate warming on flowering plants and insect pollinators and potential consequences for their interactions. Curr. Zool. 59, 418–426.

Schiappacasse, F., PeñaililO, P., Yáñez, P., Bridgen, M., 2005. Propagation studies on Chilean geophytes. Acta Hortic. 673, 121–126. doi:10.17660/ActaHortic.2005.673.13.

Silvertown, J., Servaes, C., Biss, P., Macleod, D., 2005. Reinforcement of reproductive isolation between adjacent populations in the Park Grass Experiment. Heredity 95, 198–205.

Stearns S.C., 1992. The evolution of life histories. Oxford University Press, UK.

Szõllõsi R., Medvegy, E., Benyes, A., 2011. Flowering phenology, floral display and reproductive success of *Iris sibirica*. Acta Bot. Hung. 53, 409–422. doi:10.1556/ABot.53.2011.3-4.20.

Therneau, T.L.T., 2009. Survival: survival analysis, including penalized likelihood. R package, version 2.35. Vienna: R Foundation for Statistical Computing: Vienna. https://cran.r-project.org/web/packages/survival/survival.pdf. (accessed 01.07.2016).

Volis, S., Blecher, M., Sapir, Y., 2010. Application of complex conservation strategy to *Iris atrofusca* of the Northern Negev, Israel. Biodivers. Conserv. 19, 3157–3169. doi:10.1007/s10531-010-9883-0

Watkinson, A.R., White, J., 1986. Some life-history consequences of modular construction in plants. Philos. Trans. R. Soc. London B 313, 31–51. DOI: 10.1098/rstb.1986.0024.

Weiner, J., 2004. Allocation, plasticity and allometry in plants. Perspect. Plant Ecol. Evol. Syst. 6, 207–215. doi:10.1078/1433-8319-00083.

Wilson, M.C., Chen, X.Y., Corlett, R.T., Didham, R.K., Ding, P., Holt, R.D., Holyoak, M., Hu G., Hughes, A.C., Jiang, L., Laurance, W.F., Liu, J., Pimm, S.L., Robinson, S.K., Russo, S.E., Si, X., Wilcove, D.S., Wu J., Yu, M., 2016. Habitat fragmentation and biodiversity conservation: key findings and future challenges. Landscape Ecol 31, 219–227. doi: 10.1007/s10980-015-0312-3.

Worley, A.C., Barrett, S.C.H., 2000. Evolution of floral display in *Eichhornia paniculata* (Pontederiaceae): direct and correlated responses to selection on flower size and number. Evolution 54, 1533–1545. doi:10.1111/j.0014-3820.2000.tb00699.x.

Zar, J.H., 1999. Biostatistical Analysis. Prentice Hall, New Jersey.

Capítulo 3

Fifteen Microsatellite Markers for *Herbertia zebrina* (Iridaceae): An Endangered Species from South American Grasslands

Cristiane Forgiarini[2,5], Manuel Curto[3], Eudes Maria Stiehl-Alves[2], Christian Bräuchler[4], Johannes Kollmann[4], Harald Meimberg[3] and Tatiana Teixeira de Souza-Chies[2]

[2] Department of Biosciences, Programa de Pós-Graduação em Botânica, Universidade Federal do Rio Grande do Sul, Brazil

[3] Institute of Integrative Nature Conservation Research, University of Natural Resources and Life Science, Vienna, Austria

[4] Department of Ecology & Ecosystem Management, Chair of Restoration Ecology, Technical University of Munich, Germany

Email addresses: crisforgiarini@gmail.com

manuelantoniocurto@gmail.com

emstiehl.alves@gmail.com

c.braeuchler@tum.de

jkollmann@wzw.tum.de

meimberg@boku.ac.at

tatiana.chies@ufrgs.br

[1] Manuscript received 14 April 2017; revision accepted 23 April 2017.

[5] Author for correspondence: crisforgiarini@gmail.com

Article published *in* Applications in Plant Sciences; May 2017; DOI: 10.3732/apps.1700035

Abstract

• Premise of the study: Polymorphic microsatellite loci were developed and used to genotype individuals of *Herbertia zebrina* (Iridaceae) as a first step for assessment of intraspecific genetic diversity.

• Methods and Results: Primer pairs for 47 markers were developed: 20 from a microsatellite-enriched library and 27 from a next-generation sequencing run using the Illumina MiSeq platform. Of those, 15 loci were considered successful, of which 12 were polymorphic and three were monomorphic. The primers were tested in 50 individuals from three populations of *H. zebrina*. Two to 14 alleles per locus were identified, and observed and expected heterozygosity were 0.00–0.95 and 0.18–0.89, respectively. Tests of cross-amplification to evaluate the applicability of these markers showed positive results in one congeneric species, *H. darwinii*, and in a phylogenetically closely related species, *Calydorea crocoides*.

• Conclusions: These microsatellite markers can be used for studies of genetic variation and genetic population structure, as well as to support conservation efforts.

Key words: *Calydorea crocoides*; *Herbertia darwinii*; *Herbertia zebrina*; Illumina MiSeq; Iridaceae; next-generation sequencing; simple sequence repeat (SSR) marker.

Introduction

Herbertia zebrina Deble (Iridaceae) is a critically endangered species of the southern Brazilian grasslands with a range of <100 km^2, high fragmentation, and declining habitat

quality (International Union for Conservation of Nature [IUCN] criterion B1ab[iii,v]). The populations are restricted to a mountainous region with granitic soils, and it was recognized as a distinct species only recently (Deble, 2010). Information on distribution, number of populations, and reproduction of *H. zebrina* is sparse (C. Forgiarini, Universidade Federal do Rio Grande do Sul, unpublished manuscript).

All known populations are located within an area that has changed substantially during the past 10 years and is severely threatened by monocultures (Roesch et al., 2009). The genus *Herbertia* Sweet is of recent origin (Goldblatt et al., 2008), and its radiation was probably linked to pollinator shifts that occur frequently in Iridaceae (Chauveau et al., 2012). Most *Herbertia* species, with the exception of the widespread *H. lahue* (Molina) Goldblatt, are restricted to South American grasslands. *Herbertia zebrina* is thus a suitable model to understand the mechanisms that lead to the high level of endemism in that region, and to study the effects of land-use changes threatening this diversity.

Microsatellite markers (simple sequence repeats [SSRs]) are a well-established approach to evaluate genetic diversity of populations for conservation planning of threatened species (Wan et al., 2014). Thus, we developed markers for *H. zebrina* using two methods of microsatellite development. In the future, we expect that these markers can be used to analyze the genetic structure of the species. We also present the conditions for amplification, primer sequences, size range, heterozygosity, Hardy–Weinberg equilibrium (HWE), null alleles, and linkage disequilibrium. To evaluate the applicability of these markers, cross-amplification was tested for the congeneric species *H. darwinii* Roitman & J. A. Castillo and for a species of another closely related genus, *Calydorea crocoides* Ravenna

Methods and Results

Total genomic DNA was extracted from silica gel–dried leaves of 50 individuals from three populations of *H. zebrina* (Appendix 1) using the cetyltrimethylammonium bromide (CTAB) protocol developed by Doyle and Doyle (1987), with modifications to the quantity of dried leaves used (10–50 mg) and microcentrifuge tube size (2-mL tubes). Two types of libraries were prepared, one using the method of Billote et al. (1999) and another using two partial (2%) Illumina MiSeq paired-end runs with read length of 300 bp (Illumina, San Diego, California, USA). For the first library, 20 primer pairs were designed from a single individual (voucher no. ESC421, Herbarium of the Universidade Federal do Rio Grande do Sul [ICN], Porto Alegre, Rio Grande do Sul, Brazil; Appendix 1). Total DNA was digested with RsaI (Invitrogen, Carlsbad, California, USA) and ligated to the adapters M28 (5'-CTCTTGCTTGAATTCGGACTA-3') and M29 (5'-TAGTCCGAATTCAAGCAAGAGCACA-3') using T4 DNA ligase. Linker-adapted fragments were then enriched by hybridization with 5' biotin (GT)8 and (CT)8 biotin-linked probes followed by purification with paramagnetic beads (Streptavidin MagneSphere Paramagnetic Particles; Promega Corporation, Madison, Wisconsin, USA). After the process described above, the enriched genomic DNA fragments were cloned into plasmid (pGEM-T Easy Vector, Promega Corporation) and single colonies containing microsatellite markers were identified by dot blot hybridization. Inserts were amplified with universal primer M13, treated with exonuclease I and shrimp alkaline phosphatase (New England Biolabs, Ipswich, Massachusetts, USA), and sequenced using the ABI 3500xL sequencer (Life Technologies/Applied Biosystems, Foster City, California, USA). Primers were designed using Primer3 (Untergasser et al., 2012), according to the following criteria: (i) size of primers 18–22 bp, (ii) melting temperature (Tm) 45–60°C, (iii) Tm

difference between primer pairs no higher than 3°C, (iv) GC content 40–60%, (v) no complementarity between primer pairs, and (vi) amplified product length 100–300 bp.

To increase the number of polymorphic loci, we also used one sample of *H. zebrina* (voucher no. CF115 [ICN]; Appendix 1) to construct an Illumina library and identify microsatellites, from which 27 primer pairs resulted. The library was sequenced twice on a MiSeq run in five steps: DNA fragmentation, end repair, dA-tailing, Y-adapter ligation, and index PCR and bioinformatics analyses according to Deck et al. (2016). This process was developed at the Institute for Integrative Nature Conservation Research, University of Natural Resources and Life Sciences (Vienna). The Illumina run was done by the Genomics Service Unit from Ludwig-Maximilians-University (Munich). Primers were designed using Primer3Plus (Untergasser et al., 2007). Fluorescent dyes were added to the primers using the M13-tailed primer method (Schuelke, 2000). Four tail primers were used, and each one was tagged with a unique fluorescent dye: 6-FAM (TGTAAAACGACGGCCAGT), VIC (TAATACGACTCACTATAGGG), NED (TTTCCCAGTCACGACGTTG), and PET (GATAACAATTTCACACAGG). The amplifications were done by multiplex, with a combination of two to four primers using HotStarTaq Plus Master Mix Kit (QIAGEN, Hilden, Germany), following the protocol described in Deck et al. (2016).

The conditions of PCR amplification were identical in both techniques, i.e., an initial denaturation at 95°C for 15 min; followed by 10 cycles of 95°C for 30 s, annealing temperature (with a touchdown of 65–60/62–58°C, –0.5°C per cycle) for 45 s, and 72°C for 30 s; 35 cycles at 95°C for 30 s, annealing temperature (58–60°C) for 45 s, and 72°C for 30 s; and a final extension at 72°C for 10 min. Of the 47 primer pairs developed from the two libraries, 33 primer pairs resulted in PCR-amplified products, six using the method of Billote

et al. (1999) and 27 using Illumina MiSeq. The amplifications were confirmed by gel electrophoresis (1.5%). One microliter of fluorescent PCR product was added into the mixture with 11 µL of formamide and 0.11 µL of GeneScan 500 LIZ Size Standard (Applied Biosystems/Life Technologies, Waltham, Massachusetts, USA). The material was sent to the Genomics Service Unit (Ludwig-Maximilians-University) for genotyping. The genotypes were analyzed using the program GeneMarker 1.75 (SoftGenetics, State College, Pennsylvania, USA). Of the 33 markers, 12 were considered polymorphic, three monomorphic (Table 1), and 18 presented poor amplification and were not included here.

To estimate the number of alleles, observed heterozygosity (Ho), expected heterozygosity (He), and HWE, we used the package pegas (Paradis, 2010) of R software version 3.2.2 (R Development Core Team, 2016). The presence of null alleles was checked using MICRO-CHECKER 2.2.3 (van Oosterhout et al., 2004), and their statistical significance was assessed using Bonferroni-corrected P values. Linkage disequilibrium was estimated using GENEPOP software version 4.2 (Rousset, 2008). The number of alleles ranged from two to 14 per locus across the three populations (Table 2), Ho was 0.00–0.95, and He was 0.18–0.89.

Overall, Ho was lower than He in the three populations, resulting in deviations from HWE for most markers. Null alleles were observed in nine loci (Table 2).

Table 1. Characteristics of 12 polymorphic and three monomorphic loci designed for *Herbertia zebrina*.

Locus	Primer sequences (5'-3')	Fluorescent dye	Repeat motif	Allele size range (bp)	T_a (°C)	GenBank acession no.
HZ2[a]	F:GCCATGGTCAAGGGAATAAG R:GGTCGCCTTCATATGCTGTT	VIC	(GA)17	208	60	KY775362

HZ3[a]	F:ACATAAAACCGGAGGGAGCA R:AACACTGGTGTGTGACATGTGTA	NED	(CA)8tgt(AC)8	180	60	KY775363
HZ4[b]	F:TAGGCCCCACCAACGTATAA R:AACACGTCATCTTCGTCTTCC	PET	(GA)10	202	60	KY775364
HZ5[b]	F:TTGGGTTGTATCTCGTATCTGG R:TGCCATGTACATCCCTTAAATC	FAM	(GC)8(AC)12(AG)17	253	60	KY775365
HZ6[a]	F:AATGCCTTTGACTGCTGACC R:GTTTGTATCGCCGAACTCGT	VIC	(GT)4gg(GT)8	158	60	KY775366
HZ7[b]	F:TGAAAGCATGATGCATAGGA R:AGGCTTGTGAATTGGGATTG	FAM	(GT)8(AG)3(AT)3	162	60	KY775367
HZ8[b]	F:TCGAGAGGGTTAGGGTTTGA R:CAAGCTCCTCCAAAGGCTATT	FAM	(GAA)7	174	60	KY775368
HZ9[b]	F:GAAGAGAATTTATGGGGCACA R:GACCCCACTTGTGGAATATCA	PET	(CAA)16	153	60	KY775369
HZ10[b]	F:GACTCGTTAAGAGAATCGAGCTT R:AATGTCATGGCTTCTTTTAGGG	NED	(GAGCC)3	151	60	KY775370
HZ10E[b,c]	F:TTCGTTGGAGTAACAGAGGACA R:CACCAAATTAGCAACCATCTGA	FAM	(TG)6	207	57	KY781890
HZ11[b]	F:TTTTGAACTGGAGGACCACA R:TTCCAAACCGTAGAGATTCCA	PET	(GAAAGA)5	177	60	KY775371
HZ12[b]	F:CAATTCTGCACCTGTACCCATA R:TGTGTGCATGCCATTTACCT	NED	(TA)15	109	60	KY775372
HZ13[b]	F:GGTTTCAGGGTTTAGGTTAGGG R:CATGAACGAACTGTCTTAGTTTGG	FAM	AT(6)GT(10)	116	60	KY775373
HZ14[b]	F:AGGTGGGTTCACCTTAAAAGA R:CATCCTATGTGGCTAGTAATGTGG	NED	(GAA)10	100	60	KY775374
HZ15[b]	F:CCAGACCTCACTCGTAGGAAAT R:TGTACCATTACCAAGAAGCAAGC	PET	(GTT)11	100	60	KY775375

Note: T_a = annealing temperature.

[a]Monomorphic markers.

[b]Tested for polymorphism.

[c] Loci developed using method of Billote et al. (1999).

Table 2. Genetic characterization of 12 newly developed polymorphic microsatellites of *Herbertia zebrina*.[a]

	Cachoeira, Brazil (n = 20)					Santana, Brazil (n = 15)					Encruzilhada, Brazil (n = 15)			
Locus[b]	A	Ho	He	HWE	Locus	A	Ho	He	HWE	Locus	A	Ho	He	HWE
HZ4	13	0.65	0.88	0.000*	HZ4	6	0.53	0.71	0.028	HZ4	3	013	0.55	0.000*
HZ5	13	0.20	0.80	0.000*	HZ5	14	0.73	0.89	0.003	HZ5	14	0.53	0.84	0.000*
HZ7	8	0.95	0.80	0.159	HZ7	9	0.93	0.83	0.115	HZ7	11	0.87	0.74	0.087
HZ8	5	0.10	0.62	0.000*	HZ8	3	0.00	0.59	0.000*	HZ8	5	0.00	0.74	0.000*

HZ9	8	0.40	0.80	0.000*	HZ9	4	0.00	0.51	0.000*	HZ9	6	0.27	0.52	0.000*
HZ10	12	0.20	0.83	0.000*	HZ10	9	0.27	0.79	0.000*	HZ10	8	0.27	0.78	0.000*
HZ10E	3	0.45	0.36	0.646	HZ10E	4	0.27	0.66	0.001	HZ10E	3	0.07	0.18	0.038
HZ11	2	0.00	0.38	0.000*	HZ11	2	0.00	0.23	0.002	HZ11	3	0.27	0.60	0.001
HZ12	6	0.00	0.65	0.000*	HZ12	8	0.13	0.61	0.000*	HZ12	4	0.07	0.24	0.001
HZ13	8	0.45	0.49	0.250	HZ13	6	0.20	0.52	0.002	HZ13	8	0.27	0.54	0.003
HZ14	12	0.70	0.86	0.000*	HZ14	8	0.33	0.83	0.000*	HZ14	9	0.53	0.76	0.000*
HZ15	10	0.40	0.86	0.000*	HZ15	9	0.73	0.81	0.335	HZ15	5	0.33	0.60	0.006
Mean	8.3	0.37	0.69	–	Mean	6.83	0.34	0.66	–	Mean	6.5	0.30	0.59	–

Note: A = number of alleles; H_e = expected heterozygosity; H_o = observed heterozygosity; *HWE* = P values of the exact test of Hardy-Weinberg equilibrium; *n* = number of individuals sampled.

[a] See Appendix 1 for geographic locations of all populations sampled.

[b] Significant presence of null alleles (HZ4, HZ5, HZ7, HZ9 and HZ15 from Cachoeira; HZ4, HZ9, HZ10, HZ12 and HZ14 from Santana; HZ4, HZ9, HZ13 and HZ15 from Encruzilhada).

* Locus showed significant deviations from Hardy-Weinberg equilibrium, after Bonferroni correction ($P < 0.001$).

Significant linkage disequilibrium was not detected after Bonferroni correction. Tests of cross-amplification using the same amplification conditions as for *H. zebrina* with the 12 polymorphic markers showed that nine of them amplified for *H. darwinii* and five for *C. crocoides* (Table 3).

Table 3. Amplification of 12 polymorphic microsatellite loci developed for *Herbertia zebrina* for one congeneric species and another genus phylogenetically close related.

Locus	*Herbertia darwinii* (*n* = 5)	*Calydorea crocoides* (*n* =5)
HZ4	+	+
HZ5	+	+
HZ7	+	+
HZ8	+	-
HZ9	+	+
HZ10	+	-
HZ10E	+	-
HZ11	-	-
HZ12	-	-
HZ13	-	+

HZ14	+	-
HZ15	+	-

Note: + = primers successfully amplified; - = primers could not be amplified.

Conclusions

The 15 microsatellites presented here are the first markers developed specifically for H. zebrina. Although three of them were determined to be monomorphic, cross-amplification testing showed that those microsatellites amplified not only for a congeneric species but also for a species in a related genus. Thus, they can be considered reliable markers and also a valuable resource for designing appropriate conservation strategies for this South American grassland species.

Acknowledgements

The authors thank laboratory technician Holger Paetsch for assistance in the molecular laboratory. Financial support was received from Deutscher Akademischer Austauschdienst (DAAD) and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) (to C.F.), the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq; grant no. 304197/2012-2 to T.T.S.C.), the German Research Foundation (grant no. KO1741/3-1 to J.K.), and the Fundação Grupo Boticário de Proteção a Natureza (project 1018/20142).

Literature cited

BILLOTE, N., P. J. L. LAGODA, A. M. RISTERUCCI, AND F. C. BAURENCS. 1999. Microsatellite enriched libraries: applied methodology for the development of SSR markers in tropical crops. *Fruits* 54: 277–288.

CHAUVEAU, O., L. EGGERS, T. T. SOUZA-CHIES, AND S. NADOT. 2012. Oil-producing flowers within the Iridoideae (Iridaceae): evolutionary trends in the flowers of the New World genera. *Annals of Botany* 110: 713–729.

DEBLE, L. P. 2010. *Herbertia zebrina* (Iridaceae, Tigridieae, Cipurinae). A new species from Rio Grande do Sul State (Brazil). *Darwiniana* 48: 93–96.

DECK, L. M. G., J. C. HABEL, M. CURTO, M. HUSEMANN, S. STURM, A. GARITANO-ZAVALA, AND H. MEIMBERG. 2016. New microsatellite markers for two sympatric Tinamou species, the Ornate Tinamou (*Nothoprocta ornata*) and Darwin's Nothura (*Nothura darwinii*). *Avian Biology Research* 9: 139–146.

DOYLE, J. J., AND J. L. DOYLE. 1987. A rapid DNA isolation procedure for small quantities of fresh leaf tissue. *Phytochemical Bulletin* 19: 11–15.

GOLDBLATT, P., A. RODRIQUEZ, M. P. POWELL, T. J. DAVIES, J. C. MANNING, M. V. D. BANK, AND V. SAVOLAINEN. 2008. Iridaceae 'Out of Australasia'? Phylogeny, biogeography, and divergence time based on plastid DNA sequences. *Systematic Botany* 33: 495–508.

PARADIES, E. 2010. pegas: an R package for population genetics with an integrated-modular approach. *Bioinformatics* 26: 419–420.

R DEVELOPMENT CORE TEAM. 2016. R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing. Version 3.3.2. Vienna, Austria. Website https://www.R-project.org. [accessed 20 January 2017].

ROESCH, L. F. W., F. C. B. VIERA, V. A. PEREIRA, A. L. SCHÜNEMANN, I. F. TEIXEIRA, A. J. T. SENNA, AND V. M. STEFENON. 2009. The Brazilian Pampa: A fragile biome. *Diversity* 1: 182–198.

ROUSSET, F. 2008. GENEPOP'007: A complete reimplementation of the GENEPOP software for Windows and Linux. *Molecular Ecology Resources* 8: 103–106.

SCHUELKE, M. 2000. An economic method for the fluorescent labelling of PCR fragments. *Nature Biotechnology* 18: 233–234.

UNTERGASSER, A., H. NIJVEEN, X. RAO, T. BISSELING, R. GEURTS, AND J. A. M. LEUNISSEN. 2007. Primer3Plus, an enhanced web interface to Primer3. *Nucleic Acids Research* 35: 71–74.

UNTERGASSER, A., I. CUTUTACHE, T. KORESSAAR, J. YE, B. C. FAIRCLOTH, M. REMM, AND S. G. ROZEN. 2012. Primer3 – new capabilities and interfaces. *Nucleic Acids Research* 40: e115.

VAN OOSTERHOUT, C., W. F. HUTCHINSON, D. P. M. WILLIS, AND P. F. SHIPLEY. 2004. Micro-checker Version 2.2.1. Website http://www.microchecker.hull.ac.uk [accessed January 2017].

WAN, J., C. WANG, J. YU, S. NIE, S. HAN, Y. ZU, C. CHEN, J. LIU, Q. WANG. 2014. Model-based conservation planning of the genetic diversity of *Phellodendron amurense* Rupr due to climate change. *Ecology and Evolution* 4: 2884–2900.

Appendix

Appendix 1. Location information for the populations of *Herbertia zebrina*, *H. darwinii* and *Calydorea crocoides* used in this study.

Species	Locality	*n*	Geographic coordinates[a]	Voucher no[b]
Herbertia zebrina Deble	Santana da Boa Vista/RS, Brazil	15	30º18'47.44"S, 52º53'24.45"W	CF107
	Cachoeira do Sul/RS, Brazil	20	30º42'44.09"S, 52º58'27.91"W	CF108
	Encruzilhada do Sul/RS, Brazil	15	30º23'45.18"S, 52º38'22.16"W	CF109
	Encruzilhada do Sul/RS, Brazil	1	30°46'20.56"S, 53°08'17.10"W	CF115c
	Encruzilhada do Sul/RS,	1	30°31'3.9"S,	ESC421

	Brazil		52°41’48.9”W	
Herbertia darwinii Roitman & J. A. Castillo	Santana do Livramento/RS, Brazil	5	30°52’28.95”S, 55°28’54.02”W	ESC502
Calydorea crocoides Ravenna	Bom Jesus/RS, Brazil	5	28°28’53.23”S, 50°19’48.67”W	ESC684

Note: *n* = number of individuals sampled; RS = Rio Grande do Sul.

[a]Datum: World Geodetic System 1984 (WGS84).

[b]All vouchers were deposited in the Herbarium of the Institute of Natural Sciences (ICN), Universidade Federal do Rio Grande do Sul, Porto Alegre, Brazil.

[c]Sample used to construct the Illumina library.

Capítulo 4

To be submitted to *Conservation Genetics*

Conservation genetics of *Herbertia zebrina* (Iridaceae) – effects of inbreeding, mating system and fragmentation of an endemic species in South American grasslands

Cristiane Forgiarini[1,2*], Christian Bräuchler[2], Eudes Maria Stiehl-Alves[1], Harald Meimberg[3], Manuel Curto[3], Johannes Kollmann[2] & Tatiana Teixeira de Souza-Chies[1]

[1] Graduate Program in Botany, Universidade Federal do Rio Grande do Sul, Porto Alegre, Brazil

[2] Chair of Restoration Ecology, Department of Ecology & Ecosystem Management, Technical University of Munich, Germany

[3] Institute of Integrative Nature Conservation Research, University of Natural Resources and Life Science, Vienna, Austria

*Correspondence author: crisforgiarini@gmail.com

Abstract

Among the factors negatively affecting biodiversity, habitat fragmentation can most strongly interfere with gene flow leading to reduced genetic diversity. This has resulted in many species being on the verge of extinction, especially in global biodiversity areas such as Southern Brazil. In order to effectively conserve threatened species it is crucial to have a good knowledge of their genetic diversity as affected by habitat fragmentation. We investigated the perennial plant *H. zebrina*, a critically endangered endemic species of

grasslands of southern Brazil, a region with a long history of habitat fragmentation due to land-use change. In total, nine populations and 140 individuals of this species were sampled. Twelve microsatellite loci were used to evaluate the genetic diversity and structure of the species. We also investigated its mating system and identified the main pollinators. The genetic data showed that observed heterozygosity was always lower than expected, and that F_{is} had positive values. A STRUCTURE analysis of the SSR data identified the existence of three genetic clusters (K = 3) and the number of groups checked by DPCA analysis. AMOVA evidenced that higher variation occurred within populations (82.0%) than between them (14.4%), and that gene exchange among populations was low. Nonetheless, the populations showed no isolation-by-distance pattern and also did not significantly differ across the distribution area according to the Mantel test. Allele frequency distribution in BOTTLENECK 1.2.02 discriminated five bottlenecked populations from nine studied. Besides observed heterozygosity was lower than expected and positive F_{is} values indicated some inbreeding, our observations of mating system identified *H. zebrina* as an obligate outcrossing species. Our results suggest that to conserve genetic diversity of the species at least some populations from the three main lineages identified should be preserved.

Keywords: Endemic; microsatellite markers; outcrossing; SSR.

Introduction

Habitat fragmentation is one of the main drivers of species extinction worldwide (Wilson, 2016). The loss of habitats in consequence of human activities affects biodiversity directly, and at the same time, reduces population viability indirectly (Fahrig, 2003; Wilson, 2016).

Several studies have shown that in small populations, evolutionary forces are more likely to acting (Wright, 1969; Franklin, 1980; Barrett and Husband 1990; Byer and Waller, 1999; Waples, 2002). In addition, stochastics effects may increasingly change allelic composition of species over time (Wright 1931; Barrett and Kohn, 1991; Melbourne, 2012). This leads to losses of genetic diversity with potentially drastic consequences for allelic variation, especially in small populations (Allendorf and Luikart, 2007). In those populations the levels of heterozygosity over generations can be reduced, and the similarities among fitness traits can increase in consequence of inbreeding (Waples, 2002; Keightley and Eyre-Walker 2007; Charlesworth and Willis, 2009).

Many studies have pointed out the relationship between the effects of inbreeding and losses of genetic diversity per generation (Brito 2009; Montgomery 2000; Charlesworth and Charlesworth, 1990). This process is considered to be directly linked with the mating system and the gene flow among populations. Deficit of heterozygotes in populations have been frequently observed in self-fertilization species (hereafter 'selfing') (Charlesworth and Charlesworth, 1987; Charlesworth and Wright 2001). It is one of the reasons why in the literature selfing in angiosperm is considered an evolutionary dead end (Wright et al. 2013). As well as, species tend to evolve from outcrossing to selfing (Stebbins, 1957; Morgan, 2001; Scofield and Schultz, 2006). On the other hand, studies have shown that outcrossing is not always a good deal for species, especially if populations are isolated and difficult pollinators exchange, which can lead to reduced gene flow among populations (Lloyd, 1987). Detecting how far the gene pool of a given species has been affected by habitat loss and isolation of populations is crucial to conservation management planning.

The grasslands of southern Brazil include ecosystems with high numbers of plant species (Boldrini, 2009), where many are considered endemic (Iganci et al., 2011). Moreover, this region has been suffering severe losses, land-use change (tree plantation and arable land use), and application of fire and fertilizers (Andrade et al., 2016). Until recently only very little information on species' richness and the composition of these grasslands was availabled (Overbeck, 2007). However, over the past few years studies have shown that this region has a high diversity of species (Souza-Chies, 2012; Pasini and Ritter, 2012; Aita et al., 2014; Carneiro, et al., 2015). On the other hand, studies evaluating genetic diversity of populations are recent (e.g. Silva et al., 2016; Turchetto et al., 2016). The region where this study was conducted has been suffering from habitat fragmentation (Oliveira, 2017). In this sense, in order to avoid losses of species due to grassland degradation and fragmentation, more population biology studies are necessary, especially concerning endangered species.

In this study we used *H. zebrina* Deble as a study model, an endemic species of South American grasslands described recently (Deble, 2010). Our question was to evaluate if reproduction among population of this species has been negatively affected by habitat fragmentation. We focused on one area that has been strongly fragmented by pine and eucalyptus plantations over the past ten years (Binkowski, 2009). To answer this question we used microsatellite markers to identify the genetic structure and the diversity of *H. zebrina*. Based on these data, we tested for possible inbreeding effects and the existence of gene flow among populations. Isolation by distance also was tested to verify the genetic differenciation across populations. We correlated our findings with habitat fragmentation and observations of the mating system. These results are intended to help make predictions on the future of the species and to develop conservation management strategies.

Materials and Methods

Study Species

Herbertia zebrina is an endemic geophyte of South American grasslands (Deble, 2010). Until recently only one population had been known and the species was included in the Red List as 'critically endangered' (Red List RS, 2014). It is diploid (2n = 14; Moraes et al., 2015) without information about its mating system and pollinators. The species has floral oils in the outer and inner tepals (Chauveau et al., 2012). Comparing it with other species within the genus *Herbertia* Sweet such as, *Herbertia lahue* Molina (Goldblatt) that is selfing and widespread in South America, *H. zebrina* has not such successful distribution. However, recent data suggest that although the species is restricted to a specific part of southern Brazil, it has a distribution area far larger than initially assumed (5,000 km²). Albeit, this area comprises distinct populations separated by 5–35 km from each other (Forgiarini et al., 2017).

Study Region

The study region is covered by natural grasslands situated in the oldest geological region of Rio Grande do Sul, Brazil (30°32'22.09"S and 52°42'28.77"W; Fig. 1a). The bedrock is Pre-Cambrian granite, and the altitude varies from 100–500 m (Rambo, 1956). It is known that during the end of the Pleistocene drier periods changed the habitat composition of this region and its species' diversity (Behling and Pillar, 2007). Nowadays, the area has a very high level of endemism of plant species and a particularly high richness of Iridaceae (Iganci et al., 2011; Souza-Chies, 2012). The floristic composition of the region is mainly formed by Poaceae, Fabaceae and Asteraceae and dominated by *Paspalum notanum* Flügge, *Axonopus*

affinis Chase, *Piptochaetium montevidense* (Spreng.) Parodi, and *Desmodium incanum* (Sw.) DC (C. Forgiarini, in prep.).

Sampling and DNA Extraction

Populations of *Herbertia zebrina* were mapped between 2013 and 2014. In total, 18 populations were identified in an area of approximately 5,000 km² (Forgiarini et al., *in press*). After mapping, nine representative populations were selected for the genetic study, covering the total range of the species (Fig. 1b). In total, 140 individuals were sampled from the nine different populations (Table 1). Leaves of these populations were collected during the flowering season (September–December) and dried in silica gel for DNA extraction. For each population one voucher specimen was collected and deposited in ICN *Herbarium*, Porto Alegre, Brazil. DNA extraction was undertaken using Doyle & Doyle (1987) protocol with some modifications adapted to microcentrifuge tubes.

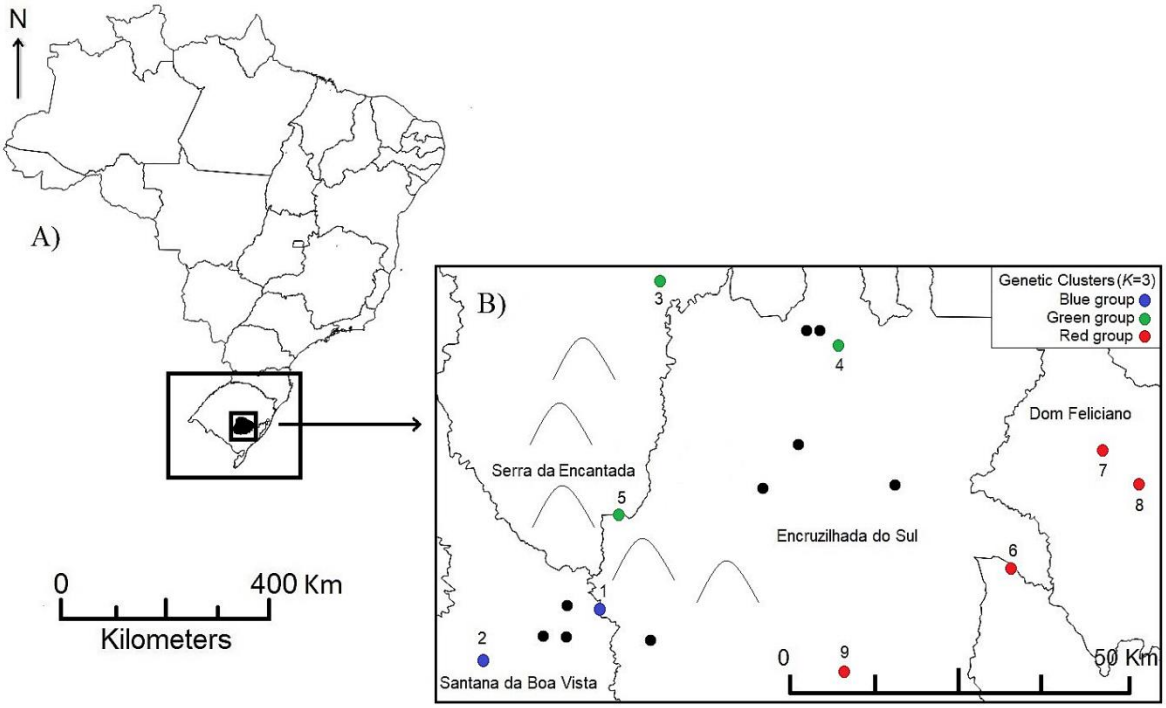

Figure 1: Location of study where the endemic grassland species *Herbertia zebrina* was mapped. Map of Brazil (A) and the 18 populations of the species identified up to date, as well as the nine populations used to analyse genetic diversity (B). The populations used in the study are differentiated by colours representing the three genetic clusters determined by STRUCTURE analysis (*K*= 3). Legend on the upper right part of the image.

*The populations 1, 2 (blue dots) are separated from others by hills (*Serrra da Encantada*) and an area considered less fragmented by forest plantation.

*Names of the main locations where the species were found: *Dom Feliciano*, *Encruzilhada do Sul* and *Santana da Boa Vista*.

Table 1: Location of the study populations of *Herbertia zebrina* in the grasslands of southern Brazil

Population	*Geographic Coordinates*[a]	*Elevation (m)*	*Population Area (ha)*	*N*	*Voucher*[b]
Pop1	*30°42'44.09"S; 52°58'27.91"W*	*165*	*2*	*15*	*CF108*
Pop2	*30°46'20.56"S; 53°08'17.10"W*	*170*	*0.3*	*15*	*CF115*
Pop3	*30°18'47.44"S; 52°53'24.45"W*	*240*	*5.5*	*20*	*CF107*
Pop4	*30°23'45.18"S; 52°38'22.16"W*	*241*	*12.7*	*15*	*CF109*
Pop5	*30°36'04.76"S; 52°56'47.27"W*	*473*	*0.4*	*15*	*CF113*

Pop6	*30°39'47.81"S; 52°23'34.94"W*	*473*	*1.5*	*15*	*CF110*
Pop7	*30°31'15.13"S; 52°15'51.22"W*	*321*	*7.8*	*15*	*CF111*
Pop8	*30°33'31.18"S; 52°12'45.80"W*	*230*	*2.7*	*15*	*CF112*
Pop9	*30°47'12.01"S; 52°37'45.10"W*	*225*	*4.9*	*15*	*CF114*

[a] World Geodetic System 1984 (WGS84);

[b] All vouchers were deposited in the Herbarium of Institute of Natural Sciences (ICN), Universidade Federal do Rio Grande do Sul, Porto Alegre, Brazil;

N = Number of individuals collected for DNA extraction.

Mating System Experiment and Pollinators Observation

During two consecutive years mating system experiments were developed in a greenhouse; 4–5 individuals of each studied population were observed to evaluate the mating system of *H. zebrina*. Four different treatments were tested: autogamy (self-fertilization); outcrossing (cross-fertilization); apomixes (asexual reproduction without fertilization) and control (without manipulation). In total 30 repetitions per treatment were performed (15 per year). The plants were isolated with individual nets from the other plants prior to the opening period. Manipulations were conducted during 9^{am} to 10^{am} after preliminary tests of stigma receptivity showing that flowers are receptive after this period. Species were observed between one to four weeks until seed production by fruits. Opening and closing times of the flowers were evaluated in the field, using 159 individuals of *H. zebrina* as a parameter. Furthermore, the main pollinators of the species were identified after approximately 200 hours of field observation. For that, were used registers of video and photo where the behaviour of the visitor were evaluated and classified as pollinator or not.

Microsatellite Markers and Genotyping

Microsatellite markers were developed using two different library methods, the enriched library of Billote et al. (1999) and two partial (2%) Illumina MiSeq (Illumina, San Diego, California, USA), as described by Forgiarini et al. (2017). In total, 12 polymorphic microsatellite markers were used to evaluate genetic diversity (loci = *HZ4*, *HZ5*, *HZ7*, *HZ8*, *HZ9*, *HZ10*, *HZ10E*, *HZ11*, *HZ12*, *HZ13*, *HZ14*, *HZ15*).

Mating System and Genetic Diversity Analysis

The mating system data, was analysed using statistic of variance (*t*-Student test) with the basic package of R 3.4.0 (R Development Core Team, 2017), to evaluate the opening and closing time of the flowers. Genetic diversity was also checked in R 3.4.0, i.e. allele number 'per locus' (*A*), heterozygosity observed (H_o), heterozygosity expected (H_e) and genetic distance value (F_{st}), using the package *Pegas* (Paradis, 2010). Probability of Hardy-Weinberg equilibrium (*pHWE*) and Linkage disequilibrium were tested within each population for each locus, respectively, in R 3.4.0. and Microchecker 2.2.3 (van Oosterhout et al., 2004). Diversity data 'per population', such as allele number (*A*), allelic richness (*Ar*), observed heterozygosity (H_o), gene diversity (H_s) and inbreeding coefficient (F_{is}) were calculated using Arlequin 3.5 (Excoffier and Lischer, 2010) and the packages *adegenet* (Jombart and Ahmed, 2011), *hierfstat* 0.04-14 (Goudet, 2005, 2014) and *devtools* (Wickham and Chang, 2017) in R 3.4.0. In order, to evaluate the existence of correlations among inbreeding coefficient (F_{is}) and population area (ha), a linear regression using basic package of R was done based on those data.

Genetic Structure and Gene Flow among Populations

Pairwise differences of F_{st} among populations were compared using the package *adegenet* in R. The matrix of Euclidean distances used in this analysis was generated in Arlequin 3.5. An Analysis of Molecular Variance (AMOVA) was performed to check the percentages of genetic variation among and between groups and/or populations (Arlequin 3.5.). We used groups to give more robustness to the AMOVA test and the results obtained (group 1 (Pop1 and Pop2), group 2 (Pop3, Pop4 and Pop5) and group 3 (Pop6, Pop7, Pop8, Pop9). The population genetic structure was analysed with Bayesian algorithms in the software STRUCTURE 2.3.4 (Pritchard et al., 2000), using 10,000 Burn-in period, 1,000000 interactions and testing population subdivision from K = 2 to K = 32. Allele frequence divergence among populations was uncorreleted. The analysis was run independent three times and K value were checked in the Structure Harvester version 0.6.7 (Earl, 2012) using ΔK method (Evanno et al., 2005). At the same time, a Discriminant Analysis of Principal Components (DAPC) was calculated using the package *adegenet* in R to check the number of groups formed of populations and to evaluate the spatial differentiation among populations.

The numbers of migrants were calculated with the software BAYESASS version 1.3 (Wilson & Rannala, 2003). Isolation by distance among populations was checked using linear regression between measures of pairwise F_{st} and geographic distances in the package *gvlma* 1.0.0.2 (Pena and Slate, 2014) in R 3.4.0. A Mantel test with 9,999 permutations was used to evaluate the existence of correlations between genetic diversity and geographic distance in R (package *adegenet*). The BOTTLENECK program version 1.2.02 (Piry et al. 1999) was used to calculate presence of bottleneck. Wilcoxon sign-rank test (Luikart et al., 1997a) was used to verify if effective size of population has remained in mutation-drift equilibrium recently. For that the two phased model of mutation (T.P.M.) were used; recommended as default of

the program. Finally, BOTTLENECK was also used to employ mode-shift indicator of the allele frequency distribution to discriminate bottlenecked population (Luikart et al, 1998).

Results

Mating System

The mating system experiment showed that the species was able to form fruits only after outcrossing treatment (in 98% of the cases). The other treatments did not present positive results or had low percentages of effectivity (Control 0%; Autogamy 2% and Apomixes 0%). Analysis of the opening and closing times of flowers showed that flowers normally open between 8 to 9 a.m. (χ^2 = 8:50 a.m.; $p < 0.001$) and close around 5 to 6 p.m. (χ^2 = 5:55 p.m.; $p < 0.001$). Furthermore, we verified that the temperature directly affected this process because in hotter days (around 40 °C) plants can close earlier than expected, at 2 p.m. (C. Forgiarini, pers. observ.). Analyses of imagens (during two consecutive years) showed the existence of nine main pollinators of *H. zebrina* (Appendix 2). Four of them were considered common pollinators. One of the bees' species collected specifically pollen (*Trigona spinipes*) and was highly efficient in the fertilization process. Other pollinators were considered also hervibores that eat flowers (e.g. *Astylus quadrilineatus)* with a high capacity to damage it.

Genetic Diversity

Genotypes based on 12 SSR markers were obtained for nine populations. The number of alleles obtained varied between 4 and 38, confirming polymorphism of the species. The average of observed heterozygosity was lower (*Ho*=0.32) than the expected values (*He*=

0.68) and ranged from 0.04 (in *HZ8*) to 0.91 (in *HZ7*) and 0.5 (in *HZ13*) to 0.89 (in *HZ5*), respectively. The average of F_{st} was equal to 0.12 and the probability of Hardy-Weinberg equilibrium in all cases were high ($p < 0.001$). The 12 microsatellites analysed for all populations together did not present significant linkage disequilibrium (See Appendix 1). The nine populations studied showed allele number (A) and allelic richness (A_r) ranged from 4.9 to 8.1 and 1.5–1.7, respectively (Table 2). Percentages of observed heterozygosity were always lower than the expected values, i.e. 0.41–0.59 for observed heterozygosity (H_o) and 0.52–0.75 for expected heterozygosity (H_e). Gene diversity (H_s) was 0.30–0.51 and the coefficient of inbreeding (F_{is}), in all cases, had positives values which indicated a deficit of heterozygotes within populations (0.27–0.35). Linear regression among F_{is} values and size of the areas showed no significant correlation ($F = 4.8$, $df = 7$, $p = 0.07$).

Table 2: Measures of genetic diversity found for the nine study populations of the grassland species *Herbertia zebrina*

Population	*Allele Number (A)*	*Allele Richness (A_r)*	H_o	H_e	H_s	F_{is}
Pop1	*6.50*	*1.66*	*0.42*	*0.71*	*0.51*	*0.35*
Pop2	*6.45*	*1.65*	*0.48*	*0.71*	*0.37*	*0.27*
Pop3	*8.09*	*1.67*	*0.46*	*0.73*	*0.43*	*0.35*
Pop4	*5.75*	*1.61*	*0.43*	*0.62*	*0.49*	*0.35*
Pop5	*8.12*	*1.50*	*0.59*	*0.75*	*0.36*	*0.30*
Pop6	*5.36*	*1.48*	*0.41*	*0.52*	*0.37*	*0.29*
Pop7	*6.09*	*1.60*	*0.42*	*0.66*	*0.38*	*0.32*
Pop8	*4.90*	*1.51*	*0.44*	*0.56*	*0.30*	*0.30*
Pop9	*5.78*	*1.50*	*0.36*	*0.61*	*0.26*	*0.34*
Mean	*5.60*	*1.57*	*0.45*	*0.57*	*0.38*	*0.31*

A, allele number; A_r, allele richness; H_o, observed heterozygosity; H_e, expected heterozygosity; H_s, gene diversity; F_{is}, inbreeding coefficient.

Values of allele frequency were found and used to calculate measures of pairwise genetic differentiation F_{st}. These data showing the genetic differentiation among the nine

populations studied, were based on Euclidean distances. Pairwise differences of F_{st} also identified that Pop2 is the more differentiated among all studied populations followed by Pop1 (Figure 2).

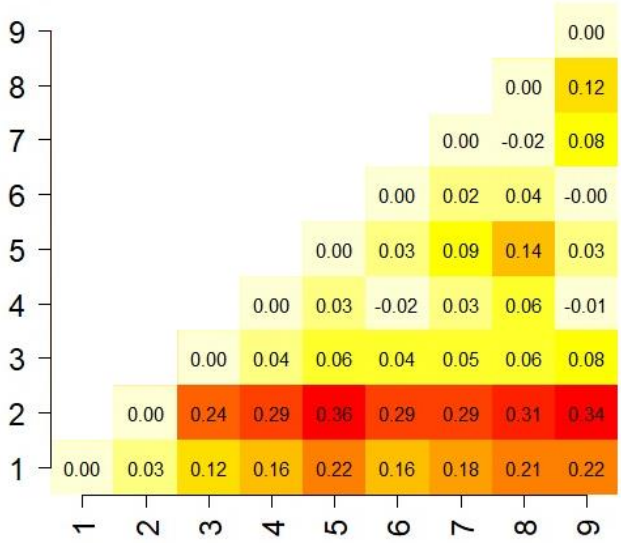

Figure 2: Pairwise differentiation matrix of F_{st} among the nine populations evaluated (The gradient of colours represents the lowest variations detected of F_{st} in white and higher variations detected in red).

The Analysis of Molecular Variance (AMOVA) of groups showed that the percentages of genetic variaton among groups were equal to 14.4% (R^2 = 17.5; *df* = 2; variance component = 0.085); 3.2% among populations within groups (R^2 = 6.5; *df* = 6; variance component = 0.01) and 82.4% within populations (R^2 = 132.5; *df* = 271; variance component = 0.48). Fixation indices of F_{st}; F_{sc} and F_{ct} were respectively 0.18, 0.04 and 0.14. The STRUCTURE analysis identified the optimal number of clusters as K = 3, indicating the presence of three main lineages. However, those lineages still share alleles in all cases (Figure 3). The first cluster containing populations is localized in the south-west region of the study area that is separated from the other populations by hills named *Serra da Encantada*. The other two

clusters are localized in a highly fragmented area represented by forest plantations and other land-use activity.

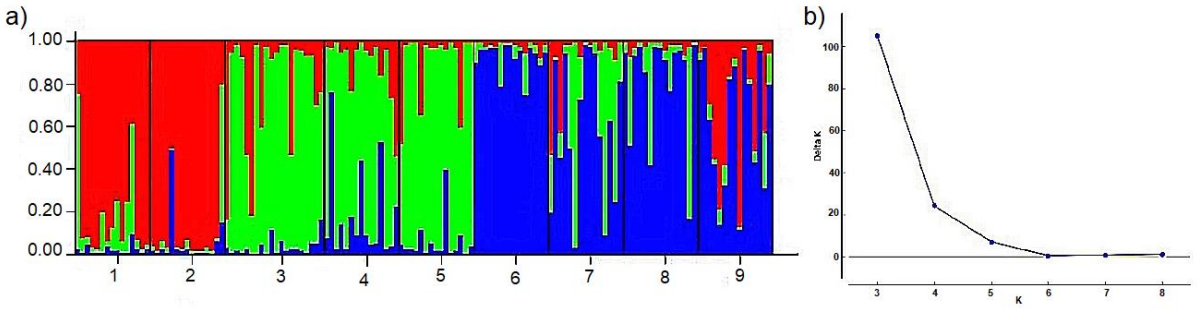

Figure 3: a) Bayesian clustering analysis of genetic differentiation in *Herbertia zebrina*, using three genetic clusters (*K* = 3) in STRUCTURE. Each column (individual) is assigned a specific suite of colours corresponding to the membership coefficient; populations are labelled according to Table 1. b) Magnitude of the modal value of *K* as a function of *K* (mean ± SD over 32 replicates).

Discriminant Analysis of Principal Components (DAPC) corroborated with the existence of three groups found in the STRUCTURE Analysis (Figure 4a). Whereas *BIC* scores of DAPC showed a declined sharply *K* = 1 to *K* = 3 and an optimal number of clusters equal to *K* = 3. Because the results corroborated with the STRUCTURE analysis, we assumed that in the best scenario possible, the species is to be separated into three groups of populations. DAPC also showed that the higher genetic variation is found along the *X* axis among cluster 2 and the other two clusters. The same analysis also showed that populations one and two are the populations that are more genetically differentiated from the other populations (Figure 4b).

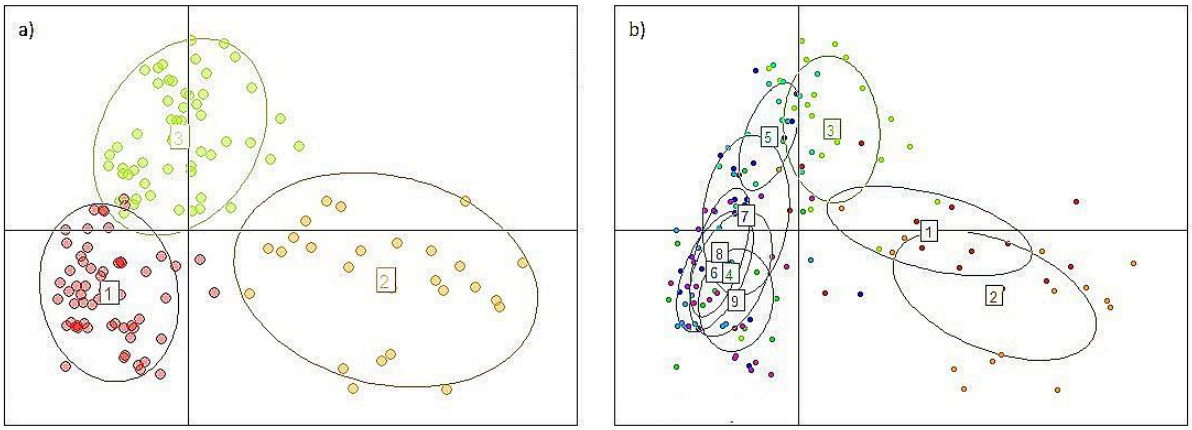

Figure 4: a) *DAPC* graphic showing the spatial differentiation among the three groups using all the 140 individuals analysed. b) Graphic showing the separation according to the nine populations identified by the DAPC analysis.

Linear regression between measures of pairwise F_{st} and geographic distances did not showed isolation by distance among populations (F = 0.054; df = 34; p = 0.81). Mantel test indicated no significant relationship among genetic differentiation and geographic distance (p = 0.34). However, migration rate in BAYESASS using a confidence interval (CI) 95% varied from 0.01 to 0.02 in the nine populations evaluated. The BOTTLENECK analysis using two phased model of mutation (T.P.M) revealed that genetic diversity loss due the reductions of effective size of populations was detected in five of nine populations studied (Pop4, Pop5, Pop6, Pop8 and Pop9). The mode-shift indicator of the allele frequency distribution to discriminate bottlenecked population revealed normal L-shaped distribution for all populations.

Discussion

Low genetic diversity has been normally associated with selfing in plants (Silvertown and Charlesworth, 2001) and not with outcrossing, which is the mating system identified for *H. zebrina*. In this sense, a successful fertilization process is essential to exchange alleles among

populations. Furthermore, species with mixed mating systems also tend to shift to outcrossing if percentages of inbreeding increased in populations (Lande and Schemske, 1985). This can happen because outcrossing species have more success establishing themselves in the field than selfing species. This is related to more efficient mechanisms for exchanging alleles as a consequence of crossing between mates (Barrett, 2014). However, in scenarios of high habitat fragmentation, pollination can be limiting for species, and in those cases, selfing would bring advantages of fruit formed (Lloyd, 1984). Furthermore, unreliable pollinators can also contribute to changes on mating system to increase fertilization successul (Opedal et al., 2016). Albeit, no all species are able to shift to selfing in situations of disturb, which could be a problem in cases of high habitat fragmentation.

Our study using microsatellites also showed that in all populations the observed heterozygosity per loci was lower than expected. Moreover, the coefficient of inbreeding revealed positive values indicating occurrence of inbreeding in the populations studied (Wright, 1931; Allendorf and Luikart, 2007). Linear regression among these variables identified that these data are not significantly correlated. The pairwise difference data of F_{st} showed low values of genetic differentiation among the populations studied, with exception of population one and two. Usually, low levels of genetic diversity in populations have been associated with restricted species distribution (Cole, 2003; Solórzano, 2016). However, this is a controversial topic in population genetics, because species with narrow distribution may also have different responses to the levels of genetic diversity (Nybom, 2004; Gibson et al. 2008).

Low levels of genetic diversity were found in other endemic species within the Iridaceae (Hannan & Orick, 2000; Kostrakiewicz & Wróblewska, 2008; Waal, 2010). In these studies,

the main reasons pointed out in order to explain this result were fragmentation, mating system of species and also capacity of recruitment compared with other grasslands. Compared with widespread species, endemics have often low genetic diversity and a high genetic structure (Loveless & Hamrick, 1984). However, studies in the genus *Herbertia* are in accordance with this theory: While the endemic *H. zebrina* with a small range has low genetic diversity the widespread *H. lahue* has better levels of genetic variation (Stiehl-Alves et al., 2016). This also was verified comparing *H. lahue* with other species of the genus *Herbertia* (Stiehl-Alves, 2017) and with the close related *Calydorea crocoides* Ravenna; (Alencar et al, in preparation)). Although *H. lahue* had a more pronounced structure among populations, and *H. zebrina* had a higher structure within populations.

Our study also showed that two populations of *H. zebrina* (population one and two) were genetically more differentiated compared with the others. These populations are situated in the least fragmented part of the species range (*database* CPRM). STRUCTURE and DPCA analysis showed the existence of three genetic lineages with shared alleles among populations. Populations are also not isolated by distance, and the actual rates of migrants among populations were considered low. In the literature, fragmentation is described as responsible for increasing the deletion effects in populations (Ellstrand and Elam 1993). Rate of migrants in the long term can contribute to increase inbreeding in species once that alleles from other populations have difficulties to establish in these populations (Bailey and McCaulley, 2006). Due this scenario of high habitat loss the species necessarily needs of an efficient pollination process to increasing gene flow among populations. The reduction of effective population size detected in BOTTLENECK analysis also needs to carefully evaluate

once that this reduction can be responsible to increase the negative effects of natural selection.

Implications for Conservation

In past decades habitat fragmentation has been reported as one of the main drivers for loss of habitats, isolation and reduction of population (Leach and Givnish, 1996). Fragmentation can divide populations into small subpopulations, effectively blocking gene flow among them (Storch, 2000). This process can also reduce the level of heterozygosity of populations and lead to inbreeding depression, genetic bottlenecks and genetic drift. In those environments, studies of genetic diversity can be used as a powerful tool for planning conservation programs, although this is still overlooked in conservation policy implementation (Laikre et al., 2010). These studies provide a possibility of mapping priority areas for conservation in order to avoid loss of allelic variation of species and ultimately extinctions (Frankham, 2010).

Our study showed the necessity the need for preservation of at least one population from the three allele lineages found for *H. zebrina*. However, to complement these analysis new studies on pollination ecology, recruitment and fitness traits would be necessary to provide that information and to select genotypes with more phenotypic plasticity aiming to increase the chances of survival of *H. zebrina*.

Acknowledgments

We thank the technician Holger Paetsch for assistance in the molecular laboratory and Gustavo Afonso Silva Arias for the insights about statistical analysis. This work was

supported by *Fundação Grupo Boticário de Proteção a Natureza* [grant number 1018/20142]. Cristiane Forgiarini thanks Capes and DAAD for the grants. Johannes Kollmann acknowledges funding by the German Research Foundation [KO1741/3-1]. Tatiana Teixeira de Souza-Chies acknowledges CNPq [grant numbers 304197/2012-2 and 304506/2016-8].

Literature cited

Andrade BO, Koch C, Boldrini, II, Vélez-Martin E, Hasenack H, Hermann JM, Kollmann J, Pillar, VD, Overbeck, GE (2015). Grassland degradation and restoration: A conceptual framework of stages and thresholds illustrated by southern Brazilian grasslands. Nat. & Conserv. 13, 95–104. doi:10.1016/j.ncon.2015.08.002.

Aita AM, Chauveau O, Souza-Chies TT, Eggers L (2014) Two new species of Sisyrinchium (Iridaceae) from Subtropical Highland Grasslands of Southern Brazil. Phytotaxa 88:9–18. doi: 10.11646/phytotaxa.88.1.3

Allendorf FW and Luikart G (2007). Conservation and the Genetics of Populations. Blackwel, Malden, Massachusetts, USA. 642 p.

Bailey MF, McCauley DE (2006) The effects of inbreeding, outbreeding and long-distance gene flow on survivorship in North American populations of *Silene vulgaris*. J Ecol 94:98–109. doi: 10.1111/j.1365-2745.2005.01090.x

Barrett SCH (2014). Evolution of Mating Systems: Outcrossing versus Selfing. (*In*) Losos J. *The Princeton Guide to Evolution* ed. Princeton: Princeton University Press, 356-362.Barrett SCH and Husband BC (1990). Variation in outcrossing rates in *Eichhornia paniculata*: the

role of demographic and reproductive factors. Plant Sp. Biol., 5, 41-55. doi: 10.1111/j.1442-1984.1990.tb00191.x

Barrett SCH and Kohn JR (1991). Genetic and evolutionary consequences of small population size in plants: implications for conservation. (*In*) Falk DA and Holsinger KE eds. *Genetics and Conservation of Rare Plants*. Oxford: Oxford University Press, 3–30.

Behling H, Pillar VD (2007) Late Quaternary vegetation, biodiversity and fire dynamics on the southern Brazilian highland and their implication for conservation and management of modern Araucaria forest and grassland ecosystems. Philos Trans R Soc B Biol Sci 362:243–251. doi: 10.1098/rstb.2006.1984

Billote N, Lagoda PJL, A. M. Risterucc AM, and Baurenc FC (1999). Microsatellite enriched libraries: Applied methodology for the development of SSR markers in tropical crops. Fruits 54: 277–288.

Binkowski P (2009). Conflitos ambientais e significados sociais em torno da expansão da silvicultura de eucalipto na "Metade Sul" do Rio Grande do Sul. PhD Thesis, Universidade Federal do Rio Grande do Sul, Brazil.

Boldrini II (2009). A flora dos campos do Rio Grande do Sul. (In) Pillar VD, Müller SC, Castilhos ZMS, Jacques AVA (Eds.), Campos Sulinos: Conservação e Uso Sustentável da Biodiversidade. Ministério do Meio Ambiente. Brasília, pp. 63-77.

Brito D (2009). Genetic consequences of population subdivision: the marsupial *Micoureus paraguayanus* (Mammalia: Didelphimorphia) as a case study. Zoologia. 26 (4): 684–693. http://dx.doi.org/10.1590/S1984-46702009000400013.

Byer DL and Waller DM (1999). Do Plant Populations Purge Their Genetic Load? Effects of Population Size and Mating History on Inbreeding Depression. Annu. Rev. Ecol. Evol. Syst. . 30:479-513.//doi.org/10.1146/annurev.ecolsys.30.1.479.

Cabreira TN, Facco MG, Miotto STS (2015). *Piriqueta pampeana*, a new species of Turneraceae (Passifloraceae s.l.) from Rio Grande do Sul, Brazil. Phytotaxa.234.1.5. doi: http://dx.doi.org/10.11646.

Charlesworth D Charlesworth B (1987). Inbreeding Depression and its Evolutionary Consequences. Annu. Rev. Ecol. Evol. Syst. 18: 237-268. https://doi.org/10.1146/annurev.es.18.110187.001321.

Charlesworth D, Charlesworth B (1990) Inbreeding Depression with Heterozygote Advantage and its Effect on Selection for Modifiers Changing the Outcrossing Rate Author (s): D . Charlesworth and B . Charlesworth Reviewed work (s): Published by : Society for the Study of Evolution Stable URL. Evolution (N Y) 44:870–888.

Charlesworth D and Willis JH (2009). The genetics of inbreeding depression. Nat. Rev. Genet. 10, 783-796 (November 2009). doi:10.1038/nrg2664.

Charlesworth D and Wright SI (2001). Breeding systems and genome evolution. Curr Opin Genet Dev. 11(6):685-90. https://doi.org/10.1016/S0959-437X(00)00254-9.

Chauveau O, Eggers L, Souza-Chies TT, Nadot S (2012) Oil-producing flowers within the Iridoideae (Iridaceae): evolutionary trends in the flowers of the New World genera. Ann Bot 110:713–729. doi: 10.1093/aob/mcs134.

Cole CT (2003). Genetic variation in rare and common plants. Annu. Rev. Ecol. Evol. Syst. 34: 213-237. https://doi.org/10.1146/annurev.ecolsys.34.030102.151717.

Cornuet JM and Luikart G (1997) Description and power analysis of two tests for detecting recent population bottlenecks from allele frequency data. Genetics 144:2001-2014.

Deble LP (2010). *Herbertia zebrina* (Iridaceae, Tigridieae, Cipurinae) – a new species from Rio Grande do Sul State (Brazil). Darwiniana 48, 93–96.

Doyle JJ and Doyle JL (1987). A rapid DNA isolation procedure for small quantities of fresh leaf tissue. Phytochemical Bulletin 19: 11–15.

Earl DA and vonHoldt BM (2012). STRUCTURE HARVESTER: A website and program for visualizing STRUCTURE output and implementing the Evanno method. Conserv. Genet. Resour. 4, 359–361. doi:10.1007/s12686-011-9548-7.

Ellstrand NC, Elam DR (1993). Population genetic consequences of small population size: implications for plant conservation. Annu. Rev. Ecol. Evol. Syst. 24:217–242.

Excoffier L and Lischer HEL (2010). ARLEQUIN suite ver 3.5: A new series of programs to perform population genetics analyses under Linux and Windows. Mol. Ecol. Resour. 10, 564–567. doi:10.1111/j.1755-0998.2010.02847.x.

Fahrig L (2003). Effects of habitat fragmentation on biodiversity. Annu. Rev. Ecol. Evol. Syst. 34:487–515. doi: 10.1146/annurev.ecolsys.34.011802.132419.

Frankham R (2010) Challenges and opportunities of genetic approaches to biological conservation. Biol Conserv 143:1919–1927. doi: 10.1016/j.biocon.2010.05.011.

Franklin IR (1980). Evolutionary change in small populations. (*In*) Soule ME, Wilcox BA eds. *Conservation Biology: An Evolutionary Ecological Perspective*. Sinauer Associates 135 – 140.

Forgiarini C, Curto M, Stiehl-Alves EM, et al (2017) Fifteen Microsatellite Markers for *Herbertia zebrina* (Iridaceae): An Endangered Species from South American Grasslands. Appl Plant Sci 5:1700035. doi: 10.3732/apps.1700035.

Gibson JP, Rice SA, Stucke CM (2008). Comparison of population genetic diversity between a rare, narrowly distributed species and a common, widespread species of *Alnus* (Betulaceae). Am. J. Bot. 95:588–596. doi: 10.3732/ajb.2007316.

Goudet J (2005). HIERFSTAT, a package for R to compute and test hierarchical F statistics. Mol. Ecol. Notes 5, 184–186. doi:10.1111/j.1471-8286.2004.00828.x.

Goudet J (2014). HIERFSTAT: Estimation and tests of hierarchical F-statistics. R package version 0.04-14. http://CRAN.R-project.org/package=hierfstat.

Iganci JRV, Heiden G, Miotto STS, Pennington RT (2011) Campos de Cima da Serra: The Brazilian Subtropical Highland Grasslands show an unexpected level of plant endemism. Bot J Linn Soc. doi: 10.1111/j.1095-8339.2011.01182.x.

Hannan GL, Orick MW (2000) Isozyme diversity in Iris cristata and the threatened glacial endemic *I. Lacustris* (Iridaceae). Am J Bot 87:293–301. doi: 10.2307/2656625.

Jombart T, Ahmed I (2011). ADEGENET 1.3-1: New tools for the analysis of genome wide SNP data. Bioinformatics 27, 3070–3071. doi:10.1093/bioinformatics/btr521.

Keightley PD, Eyre-Walker A (2007). Joint inference of the distribution of fitness effects of deleterious mutations and population demography based on nucleotide polymorphism

frequencies. Genetics.177(4):2251-61. https://doi.org/10.1534/genetics.107.080663.

Kostrakiewicz K, Wroblewska A (2008) Low genetic variation in subpopulations of an endangered clonal plant Iris sibirica in southern Poland. Ann Bot Fenn 45:186–194. doi: 10.5735/085.045.0303.

Laikre L (2010) Genetic diversity is overlooked in international conservation policy implementation. Conserv Genet 11:349–354. doi: 10.1007/s10592-009-0037-4.

Lande R and Schemske DW (1985). The Evolution of Self-Fertilization and Inbreeding Depression in Plants. I. Genetic Models Evolution. 39 :1, 24-40. doi: 10.2307/2408514.

Leach MK and Givnish TJ (1996). Ecological Determinants of Species Loss in Remnant Prairies. Science, 273, 1555-1558. http://dx.doi.org/10.1126/science.273.5281.1555.

Lloyd DG (1984). Gender allocations in outcrossing cosexual plants. (*In*) *Perspectives on Plant Population Ecology* (R. Dirzo and J. Sarukhán, eds. Sinauer, Sunderland 277–300.

Lloyd. DG 1987. Allocations to Pollen, Seeds and Pollination Mechanisms in Self-Fertilizing Plants. Funct Ecol. 1:2, 83-89.

Loveless MD, Hamrick JL (1984) Ecological determinants of genetic structure in plant populations. Annu. Rev. Ecol. Evol. Syst. 15: 65-95.

Luikart G, Allendorf FW, Cornuet JM and Sherwin WB (1997) Distortion of allele frequency distributions provides a test for recent population bottlenecks. Journal of Heredity. 1998:89(3).

Luikart G and Cornuet JM (1998) Empirical evaluation of a test for identifying recently bottlenecked populations from allele frequency data. Conservation Biology 12(1):228-237.

Melbourne BA (2012). Demographic Stochasticity. (*In*) A. Hastings and L. J. Gross eds. *Sourcebook in Theoretical Ecology*. Berkeley: University of California Press.

Montgomery ME, Woodworth LM, Nurthen RK, et al (2000) Relationships between population size and loss of genetic diversity: Comparisons of experimental results with theoretical predictions. Conserv Genet 1:33–43. doi: 10.1023/A:1010173401557.

Moraes AP, Souza-Chies TT, Stiehl-Alves EM, Burchardt P, Eggers L, Siljak-Yakovlev S, Brown SC, Chauveau O, Nadot S, Bourge M, Viccini LF, Kaltchuk-Santos E (2015) Evolutionary trends in Iridaceae: New cytogenetic findings from the New World. Bot J Linn Soc 177:27–49. doi: 10.1111/boj.12232.

Morgan, MT (2001) Consequences of life history for inbreeding depression and mating system evolution in plants. Proceedings of the Royal Society of London, B, Biological Sciences 268: 1817–1824. doi: 10.1098/rspb.2001.1741.

Nybom H (2004) Comparison of different nuclear DNA markers for estimating intraspecific genetic diversity in plants. Mol. Ecol. 13, 1143–1155. doi: 10.1111/j.1365-294X.2004.02141.x.

Oliveira TE de, Freitas DS de, Gianezini M, et al (2017) Agricultural land use change in the Brazilian Pampa Biome: The reduction of natural grasslands. Land use policy 63:394–400. doi: 10.1016/j.landusepol.2017.02.010.

Overbeck GE, Müller SC, Fidelis A, Pfadenhauer J, Pillar, VD, Blanco CC, Boldrini II, Both R, Forneck ED. 2007. Brazil's neglected biome: The South Brazilian Campos. Perspect. Plant Ecol. Evol. Syst. 9: 101–116. https://doi.org/10.1016/j.ppees.2007.07.005.

Opedal ØH, Albertsen E, Armbruster WS, Pérez-Barrales R, Falahati-Anbaran M, Pélabon C (2016) Evolutionary consequences of ecological factors: pollinator reliability predicts mating-system traits of a perennial plant. Ecol. Lett. 19: 1486–1495. doi: 10.1111/ele.12701.

Rambo B (1956) A fisionomia do Rio Grande do Sul. Universidade do Vale do Rio dos Sinos, Brazil. 3a ed., Editora Unisinos, Porto Alegre. São Leopoldo. 472 p.

R Development Core Team (2017) R: A language and environment for statistical computing. Version 3.4.0. R Foundation for Statistical Computing, Vienna, Austria. Website https://www.R-project.org [accessed 20 May 2017].

Red List RS (2014) Lista de espécies RS - Rio Grande do Sul. 2014. Decreto estadual n 52.109, publicado no DOE nº 233 em 02/12/2014. http://www.fzb.rs.gov.br/conteudo/4809. (accessed 01.06.2016).

Scofield, DG and Schultz ST (2006) Mitosis, stature and evolution of mating systems: low-ϕ and high-ϕ plants. Proceedings of the Royal Society of London, B, Biological Sciences 273: 275–282. doi:10.1098/rspb.2005.3304.

Silvertown & Charlesworth (2001) Introduction to Plant Population Biology. 4 ed. Wiley-Blackwell. 360p.

Silva LN, Essi L, Welker CAD, Souza-Chies TT (2016) Assessing the genetic diversity and population structure of the endangered *Chascolytrum bulbosum* (Poaceae, Poeae) using AFLP markers. Biochem. Syst. Ecol. 68, 236-242. https://doi.org/10.1016/j.bse.2016.07.027.

Solórzano S, Arias S, Dávila P (2016) Genetics and conservation of plant species of extremely

narrow geographic range. Diversity 8:1–12. doi: 10.3390/d8040031.

Souza-Chies TT, Kaltchuk-Santos E, Eggers L, Flores AM, Stiehl-Alves EM, Fachinetto J, Lustosa J, Corrêa LB, Tacuatiá LO, Piccoli P and Miz RB (2012) Studies on diversity and evolution of Iridaceae species in southern Brazil. Genet. Mol. Biol. 35: (4) 1027-1035. http://dx.doi.org/10.1590/S1415-47572012000600018.

Stebbins GL (1957) Self-fertilization and population variability in higher plants. Am. Nat. 91, 337–35410.1086/281999 (doi:10.1086/281999).

Stiehl-Alves EM, Kaltchuk-Santos E, Eggers L and Souza-Chies TT (2017) Using a population genetics approach for a preliminary investigation concerning species boundaries in *Herbertia* (Iridaceae). Int. J. Plant Sci. 178(6): 439-449. 2017. DOI: 10.1086/692014.

Storch D (2000). Reviewed Work(s): Metapopulation Biology: Ecology, Genetics and Evolution. (*In*) Hanski A and Gilpin ME. Folia Geobotanica. 35:3 347-349.

Turchetto C, Segatto ALA, Mäder G, Rodrigues DM, Bonatto SL and Freitas LB (2016) High levels of genetic diversity and population structure in an endemic and rare species: Implications for conservation. AoB Plants 8:1–17. doi: 10.1093/aobpla/plw002.

Paradis E (2010) pegas: An R package for population genetics with an integrated-modular approach. Bioinformatics (Oxford, England) 26: 419–420. DOI: 10.1093/bioinformatics/btp696.

Pasini E, Ritter MR (2012) *Trichocline cisplatina* (Asteraceae, Mutisieae), a new species from southern Brazil and Uruguay Most 25:19–25. DOI: http://dx.doi.org/10.11646/phytotaxa.42.1.3.

Pena, EA and Slate, EH (2006) "Global Validation of Linear Model Assumptions," J Am Stat Assoc. 101(473):341-354. doi: 10.1198/016214505000000637.

Piry S, Luikart G and Cornuet JM (1999) BOTTLENECK: a computer program for detecting recent reductions in the effective population size using allele frequency data. J Hered 90:502-503.

Pritchard JK, Stephens M, Donnelly P (2000) Inference of population structure using multilocus genotype data. Genetics 155, 945–959.

van Oosterhout C, Hutchinson WF Willis DPM, and Shipley PF (2004) MICRO-CHECKER Version 2.2.3. Website http://www.microchecker.hull.ac.uk [accessed May 2017].

Waal C De, Biology E (2010) Reproductive Ecology of Bird-Pollinated Babiana (Iridaceae): Floral Variation , Mating Patterns and Reproductive Ecology of Bird-Pollinated Babiana (Iridaceae): Floral Variation , Mating Patterns and Genetic Diversity. Master Thesis. 158 p.

Waples RS (2002) Evaluating the effect of stage-specific survivorship on the Ne/N ratio. Mol Ecol 11:1029–1037. doi: 10.1046/j.1365-294X.2002.01504.x.

Wilson GA. and Rannala B (2003) Bayesass version 1.3. Bayesian inference of recent migration rates using multilocus genotypes. Genetics 163: 1177–1191

Wickham H and Chang W (2015). devtools: Tools to Make Developing R Packages Easier. R package version 1(0): 185.

Wilson MC, Chen X-Y, Corlett RT, et al (2016) Habitat fragmentation and biodiversity conservation: key findings and future challenges. Landsc Ecol 31:219–227. doi: 10.1007/s10980-015-0312-3.

Wright S (1931) Evolution in Mendelian populations. Genetics.16:2 97-159.

Wright S (1969) Evolution the Genetics of Populations. The Theory of Gene Frequencies. Chicago: Univ. Chicago Press. 520 p.

Wright SI, Kalisz S and Slotte T (2013) Evolutionary consequences of self-fertilization in plants. *Proc R Soc B*. 280:20130133. DOI: 10.1098/rspb.2013.0133.

Appendices

Appendix 1

Table of values of genetic diversity per locus for the twelve markers used to study the genetic diversity of *Herbertia zebrina*.

Locus	*A*	*Ho*	*He*	*pHWE*	*Fst*
HZ10E[a]	*27*	*0.2*	*0.87*	*0.000*	*0.16*
HZ4[b]	*21*	*0.44*	*0.78*	*0.000*	*0.18*
HZ5[b]	*38*	*0.41*	*0.89*	*0.000*	*0.05*
HZ7[b]	*24*	*0.91*	*0.83*	*0.000*	*0.05*
HZ8[b]	*7*	*0.04*	*0.68*	*0.000*	*0.05*
HZ9[b]	*11*	*0.15*	*0.57*	*0.000*	*0.08*
HZ10[b]	*9*	*0.14*	*0.33*	*0.000*	*0.36*
HZ11[b]	*4*	*0.06*	*0.53*	*0.000*	*0.17*
HZ12[b]	*21*	*0.05*	*0.43*	*0.000*	*0.03*
HZ13[b]	*23*	*0.21*	*0.5*	*0.000*	*0.02*
HZ14[b]	*25*	*0.8*	*0.9*	*0.000*	*0.04*
HZ15[b]	*17*	*0.46*	*0.87*	*0.000*	*0.25*
Average	*18.92*	*0.32*	*0.68*	*0.000*	*0.12*

[a] *SSR marker developed using enriched library of Billote et al,(1999).*

[b] *SSR marker developed using Illumina MiSeq.*

A, number of alleles; *Ho,* observed heterozygosity; *He,* expected heterozygosity; p*HWE,* probability of Hardy-Weinberg equilibrium; *Fst,* allele frequence.

Appendix 2

List of pollinators and floral visitors identified during the study. This list of names was organized based in approximately 200 hours of observation on three natural populations of *Herbertia zebrina* (population 4, 6 and 9).

Number	Species	Author	Voucher
1	*Chalepogenus goeldianus**	Friese, 1899	CF120
2	*Lanthanomelissa betinae**	Urban, 1996	CF121
3	*Plebeia* sp.*	Schwarz, 1938	CF122
4	*Trigona spinipes**	Fabricius, 1793	CF123
5	*Cephalocolletes* sp.*	Michener, 1989	CF124
6	*Lasioglossum* sp. *(Dialictus)**	Curtis, 1833	CF125
7	*Astylus quadrilineatus*	Germar, 1825	CF125
8	*Cerphidea* sp.*	Newman, 1834	CF126
9	*Cleroidea*	Latreille, 1802	CF127

*Pollinators identified by Dr. Antônio Aguiar, department of zoology of Brasília University.

All the invertebrate were deposited in the zoological collection of UFRGS (Universidade Federal do Rio Grande do Sul, department of zoology).

Capítulo 5

Considerações finais

Diversos estudos têm apontado a necessidade de uma avaliação conjunta de aspectos ecológicos e moleculares para o entendimento dos processos pelos quais as espécies estão expostas (Hipp et al., 2015; Johnson et al., 2009). Desde a publicação de trabalhos como, por exemplo, o de Baker (2000) e/ou Silvertown & Charlesworth (2001), ficou evidente que a biologia molecular passou a ser uma ferramenta importante em estudos de populações. Por outro lado, este tipo de trabalho, especialmente com plantas, ainda são escassos no Brasil. O trabalho apresentado ao longo desta tese foi planejado com o intuito de reduzir um pouco esta lacuna e possibilitar a aplicação dos resultados encontrados em planos de manejos que sejam mais eficientes para conservação de espécies. Por isso, ao longo do estudo utilizamos como modelo a Iridaceae *Herbertia zebrina* que até então não possuía informações sobre ecologia e diversidade genética para realizar esta investigação. A espécie foi escolhida também devido ao seu aparente endemismo e distribuição restrita (Deble, 2010) visto que dentro deste pequeno gênero é possível encontrar espécies com uma ampla gama de distribuição (Goldblatt & Manning, 2008).

Durante o desenvolvimento desta Tese, buscamos entender os mecanismos reprodutivos, de diversidade genética e aspectos ecológicos envolvidos com *H. zebrina* para obter uma avaliação consistente com seu *status* atual e contribuir para a conservação da mesma.

No capítulo dois, mostramos resultados quanto à avaliação da área total de distribuição da espécie, que se fazia necessária como base para o planejamento do resto do

estudo, mas que até então ainda não era conhecida. Neste capítulo também apresentamos dados sobre tamanho populacional e número de indivíduos por m². Como características ecológicas em geral e relacionadas à aptidão reprodutiva das espécies têm sido consideradas importantes medidas associadas a níveis de diversidade genética (Waples, 2002; Charlesworth & Willis, 2009), buscamos estudar tais parâmetros para *H. zebrina*, bem como a avaliação do seu potencial de germinabilidade. Em consequência desta avaliação, importantes resultados foram encontrados, como por exemplo, o fato de termos identificado à existência de plasticidade fenotípica em algumas das características avaliadas, assim como, os altos valores de germinação da espécie.

Neste estudo, porém, os fatores determinantes para enquadrar *H. zebrina* como uma espécie criticamente ameaçada, segundo os critérios B2ab(ii,iii) (diante das ferramentas atualmente disponíveis pela instituição), foi a fragmentação de habitats e área de ocupação da mesma (IUCN, 2017). Seja como for, além de toda esta série de novas informações que foram encontradas para *H. zebrina* este trabalho trouxe à tona a discussão da utilização de dados quantitativos de biologia de populações para avaliar o *status* de conservação da espécie. Visto que uma das principais críticas dos estudiosos em biologia da conservação com relação às listas geradas é que geralmente não existe a realização de nenhum estudo de biologia de populações das espécies avaliadas para a obtenção dos resultados apresentados (Brummitt, 2008). Desta forma, erros sobre o *status* de ameaça das espécies acabam sendo recorrentes (Webb, 2008) e como discutimos ao longo do capítulo dois, são difíceis de serem mudados posteriormente.

No capítulo três apresentamos o desenvolvimento de marcadores microssatélites específicos para *H. zebrina* empregando duas diferentes técnicas. Salientamos a

necessidade da dupla preparação de bibliotecas utilizando a técnica de *Next Generation Sequencing.* Enfatizamos nesse capítulo que a obtenção destes marcadores não foi uma tarefa simples ao longo do estudo, exigindo uma grande dedicação de tempo ao longo da pesquisa desenvolvida. De qualquer forma, ao final deste trabalho sequências polimórficas foram encontradas para a espécie. Além disso, o teste de amplificação cruzada sugere que estes marcadores possam vir a ser utilizados em outras espécies relacionadas à *H. zebrina*, sendo um grande legado para os estudos em Iridaceae da região sul do Brasil, visto que até o presente momento para estas espécies, marcadores microssatélites específicos foram desenvolvidos apenas para o gênero *Sisyrinchium* (Miz et al., 2016; Tacuatiá, 2012b).

No capítulo quatro foi apresentado que *H. zebrina* realiza obrigatoriamente fertilização cruzada e que é fecundada geralmente por diferentes polinizadores. Apesar de ter sido detectada a presença de polinizadores com preferência por determinados recursos florais, o estudo mostrou que estes polinizadores identificados não são exclusivos para *H. zebrina* e que alguns deles também agem como predadores das estruturas florais das plantas. Os resultados da avaliação dos parâmetros de diversidade genética para *H. zebrina* mostraram também que a espécie possui altos níveis de endogamia, ao mesmo tempo em que apresenta baixas taxas de migração entre populações. Sabe-se que espécies de reprodução cruzada em ambientes fragmentados possuem maior dificuldade para realizar trocas gênicas do que espécies autocompatíveis (Lloyd, 1987), assim sugere-se que a fragmentação de habitats é o grande problema enfrentado atualmente para a sobrevivência e fluxo gênico das populações de *H. zebrina*. De qualquer forma, este trabalho mostrou também que apesar dos problemas atuais ligados a dificuldade de fluxo gênico entre

populações, o compartilhamento de alelos entre as três diferentes linhagens identificadas ainda existe, o que é um bom sinal para espécie.

De maneira geral, ao longo da tese foi possível identificar que a fragmentação de habitats e o consequente isolamento e redução das populações desencadeiam uma série de processos que ameaçam a sobrevivência das populações de *H. zebrina*. Por outro lado, algumas questões ainda precisam ser respondidas, como por exemplo, por que uma espécie com altos percentuais de germinabilidade não tem o mesmo sucesso de recrutamento comparado a outras espécies de *Herbertia* (Martins et al., dados não publicados). Existiria algum tipo de processo de dormência atrelado à espécie ou este resultado constitui uma decorrência da falta de competitividade com relação a outras espécies (Griggs, 1940). Outra importante questão, visto que o pastejo reduz a altura da vegetação e também pode afetar a comunidade de polinizadores (Gess & Gess, 1993) ao mesmo tempo em que a total exclusão do gado também pode ter impactos negativos para espécies de dimensões reduzidas (Bailey, 2004), seria importante verificar qual é de fato a intensidade de pastejo adequada para manter as populações de *H. zebrina* com bons índices de recrutamento. A possibilidade de acompanhar a espécie durante mais tempo também para avaliar impactos climáticos seria outro ponto a ser avaliado, visto que a diferença de 1°C da temperatura entre anos de amostragem poderia ser também uma das causas da redução da frutificação. Por fim, existem outras linhagens genéticas em *H. zebrina*, ou estas encontradas seriam as únicas existentes? E ainda, a falta de polinizadores específicos em *H. zebrina* seria uma decorrência da perda de polinizadores mais eficientes no passado o que acabou tornando uma espécie autoincompatível menos competitiva na ocupação de espaço do que espécies compatíveis do gênero e restringindo sua área de distribuição? Um grande passo foi dado

através do estudo realizado, mas como podemos ver muitas questões ainda precisam ser investigadas.

Perspectivas

Faz-se necessário a realização de estudos que avaliem a sobrevivência das espécies no período pós-germinação de sementes, pois estudos tem mostrado que mesmo espécies com alta taxa de germinação podem apresentar baixa capacidade de competição durante o período de recrutamento (Fenner & Thompson, 2005). Neste sentido, a realização de novos experimentos capazes de responder esta pergunta são essenciais. A influência do pastejo no recrutamento de novos indivíduos de *H. zebrina* também necessita ser avaliada de forma experimental, pois isso poderia auxiliar a aumentar os percentuais de frutificação da espécie. Seria importante também realizar uma comparação mais efetiva entre as três diferentes linhagens genéticas encontradas neste trabalho. Medições de novas características ecológicas da espécie e experimentos de polinização capazes de avaliar também as distâncias com as quais o polén é transportado seriam essenciais para conservar a espécie ao mesmo tempo em que, poderia se estabelecido diretrizes para auxiliar produtores rurais com relação aos limites de áreas plantadas. A ampliação dos estudos de diversidade genética para outras populações da espécie também seria interessante para aumentar nossa compreensão sobre as inter-relações genéticas presentes dentro de *H. zebrina* e conservar sua variabilidade.

Principais conclusões

1- *Herbertia zebrina* é uma espécie criticamente ameaçada presente nas pastagens naturais do sul do Brasil.

2-É uma espécie endêmica do Rio Grande do Sul (Serra do Sudeste) e com uma área de distribuição que abrange quatro diferentes municípios (Encruzilhada do Sul, Santana da Boa Vista, Amaral Ferrador e Dom Feliciano).

3-Representantes das três linhagens encontradas necessitam ser preservadas, especialmente representantes das populações nomeadas como um e dois neste trabalho, pois as mesmas possuem maiores frequências alélicas.

4-As populações de *H. zebrina* estão sofrendo processo de endogamia e as principais causas parece ser a fragmentação de habitats e a dificuldade de trocas gênicas entre populações, uma vez que, as populações situadas em áreas que não possuem um histórico tão pronunciado de fragmentação de habitats são as populações com maiores valores de diversidade genética.

5-A interdisciplinaridade só trouxe benefícios para o estudo realizado.

Literatura citada

Baker A (2000) Molecular Methods in Ecology. Wiley-Blackwell. 348 p.

Bailey DW (2004) Management strategies for optimal grazing distribution and use of arid rangelands. J Anim Sci. 82 E-Suppl:E147-153.

Brummitt N, Bachman SP, Moat J (2008) Applications of the IUCN Red List: towards a global barometer for plant diversity. Endang Species Res: 6:127–135.

Charlesworth D and Willis JH (2009) The genetics of inbreeding depression. Nature Reviews Genetics. 10: 783-796.

Deble LP (2010) *Herbertia zebrina* (Iridaceae, Tigridieae, Cipurinae) – a new species from Rio Grande do Sul State (Brazil). Darwiniana 48, 93–96.

Fenner M and Thompson K (2005) The Ecology of Seeds. Cambridge: Cambridge University Press. 260p.

Lloyd DG (1987) Allocations to Pollen, Seeds and Pollination Mechanisms in Self-Fertilizing Plants. Funct Ecol. 1:2, 83-89.

Gess FW and Gess SK (1993) Effects of increasing land utilization on species representation and diversity of aculeate wasps and bees in the semiarid areas of Southern Africa. In: LaSalle J., Gauld, L.D., (eds.) Hymenoptera and biodiversity. Wallingford: CAB International, p. 83–113.

Goldblatt, P & Manning, JC (2008) The iris family: natural history and classification. Timber Press, Portland, OR. 336p.

Griggs, RF (1940) The ecology of rare plants. Bull. Torrey Bot. Club. 67, 575–594.

Hipp AL, Larkin DJ, Barak RS, Bowles ML, Cadotte MW, Jacobi SK, Lonsdorf E, Scharenbroch BC, Williams E and Weiher E (2015) Phylogeny and ecological restoration. 102: 5 647-648.

Tacuatiá LO, Cidade FW, Souza AP and Souza-Chies TT (2012b). Development and characterization of nine microsatellite loci for *Sisyrinchium micranthum* (Iridaceae). *Am. J. Bot.* 99: e402-e404.

Miz RB, Tacuatiá LO, Cidade FW, Souza AP, Bered F, Eggers L, Souza-Chies TT (2016) Isolation and characterization of microsatellite loci in Sisyrinchium (Iridaceae) and cross amplification in other genera. Genetics and Molecular Research 16:15(3).

Silvertown & Charlesworth (2001) Introduction to Plant Population Biology. 4 ed. Wiley-Blackwell. 360p.

Waples RS(2002) Evaluating the effect of stage-specific survivorship on the Ne/N ratio. Mol Ecol 11:1029–1037.

Webb GJW (2008) The dilemma of accuracy in IUCN Red List categories, as exemplified by hawksbill turtles *Eretmochelys imbricata*. Endang Species Res. Publishing online, 24 October.

ANEXOS

Lista preliminar das espécies que ocorrem em área onde *Hebertia zebrina* foi identificada.

Espécie	Família
Aeschynomene falcata (Poir.) DC.	Fabaceae
Amaranthus sp.	Amaranthaceae
Anagallis arvensis L.	Primulaceae
Andropogon selloanus Hack.	Poaceae
Andropogon ternatus (Spreng.) Nees	Poaceae
Aristida filifolia (Arechav.) Herter	Poaceae
Aristida jubata (Arechav.) Herter	Poaceae
Aristida laevis (Nees) Kunth	Poaceae
Aristida venustula Arechav.	Poaceae
Aspilia montevidensis (Spreng.) Kuntze	Poaceae
Axonopus affinis Chase	Poaceae
Axonopus siccus (Nees) Kuhlm.	Poaceae
Baccharia sp.	Asteraceae
Baccharis articulata (Lam.) Pers.	Asteraceae
Baccharis crispa Spreng.	Asteraceae
Baccharis dracunculifolia DC.	Asteraceae
Borreria eryngioides Cham. & Schltdl.	Rubiaceae
Borreria sp.	Rubiaceae
Bulbostylis juncoides (Vahl) Kük. ex Osten	Cyperaceae

Carex phalaroides Kunth	Cyperaceae
Carex sp.	Cyperaceae
Centrosema virginianum (L.) Benth.	Fabaceae
Cerastium glomeratum Thuill.	Caryophyllaceae
Chaptalia piloselloides (Vahl) Baker	Asteraceae
Chaptalia runcinata Kunth	Asteraceae
Chascolytrum subaristatum (Lam.) Desv.	Poaceae
Chevreulia acuminata Less.	Asteraceae
Chevreulia sarmentosa (Pers.) Blake	Asteraceae
Commelina erecta L.	Commelinaceae
Conyza bonariensis (L.) Cronquist 1943	Asteraceae
Conyza primulifolia (Lam.) Cuatrec. & Lourteig	Asteraceae
Crotalaria hirsuta Willd.	Fabaceae
Cuphea glutinosa Cham. & Schltdl.	Lythraceae
Cyclospermum leptophyllum (Pers.) Sprague ex Britton & P. Wilson	Apiaceae
Cyperaceae sp.	Cyperaceae
Cyperus aggregatus (Willd.) Endl.	Cyperaceae
Cyperus reflexus Vahl	Cyperaceae
Danthonia cirrata Hack. & Arechav.	Poaceae
Desmanthus virgatus (L.) Willd.	Fabaceae
Desmodium adscendens (Sw.) DC.	Fabaceae
Desmodium incanum DC.	Fabaceae
Dichanthelium sabulorum (Lam.) Gould & C.A. Clark	Poaceae
Dichondra sericea Sw.	Convolvulaceae
Diodia sp.	Rubiaceae
Eleusine tristachya (Lam.) Lam.	Poaceae
Eragrostis lugens Nees	Poaceae
Eragrostis neesii Trin.	Poaceae
Eragrostis polytricha Nees	Poaceae
Eriosema sp.	Fabaceae
Eryngium horridum Malme	Apiaceae
Evolvulus sericeus Sw.	Convolvulaceae
Facelis retusa (Lam.) Sch. Bip.	Asteraceae
Fimbristylis sp.	Cyperaceae
Galianthe equisetoides (Cham. & Schltdl.) E.L.Cabral	Rubiaceae
Galium richardianum (Gillies ex Hook. & Arn.) Endl. ex Walp.	Rubiaceae
Galium sp.	Rubiaceae
Gamochaeta americana (Mill.) Wedd.	Asteraceae
Glandularia marrubioides (Cham.) Tronc.	Verbenaceae
Glandularia selloi (Spreng.) Tronc.	Verbenaceae
Glechon ciliata Benth.	Lamiaceae
Herbertia zebrina Deble	Iridaceae
Herbertia lahue (Molina) Goldblatt	Iridaceae
Hydrocotyle exigua Malme	Araliaceae
Hypochaeris sp.	Asteraceae
Hypoxis decumbens L.	Hypoxidaceae
Juncus capillaceus Lam.	Juncaceae
Kelissa brasiliensis (Baker) Ravenna	Iridaceae
Kyllinga odorata Vahl	Cyperaceae
Lathyrus subulatus Lam.	Fabaceae
Lucilia sp.	Asteraceae
Macroptilium prostratum (Benth.) Urb.	Fabaceae
Mecardonia tenella (Cham. & Schltdl.) Pennell	Plantaginaceae
Melica rigida Cav.	Poaceae

Melinis repens (Willd.) Zizka	Poaceae
Microchloa indica (L.f.) P. Beauv.	Poaceae
Mimosa sp.	Fabaceae
Mnesithea selloana (Hack.) de Koning & Sosef	Poaceae
Oxalis articulata Savigny	Oxalidaceae
Oxalis brasiliensis Lodd.	Oxalidaceae
Oxalis eriocarpa DC.	Oxalidaceae
Oxalis lasiopetala Zuccarini	Oxalidaceae
Oxalis sp.	Oxalidaceae
Paspalum notato Fluegge	Poaceae
Paspalum plicatulum Michx.	Poaceae
Paspalum umbrosum Trin.	Poaceae
Pavonia sp.	Malvaceae
Pfaffia tuberosa (Spreng.) Hicken	Amaranthaceae
Piptochaetium lasianthum Griseb.	Poaceae
Piptochaetium montevidense (Spreng.) Parodi	Poaceae
Piptochaetium ruprechtianum Desv.	Poaceae
Piptochaetium stipoides (Trin. & Rupr.) Hack.	Poaceae
Piriqueta taubatensis (Urb.) Arbo	Passifloraceae
Plantago myosuros Lam.	Plantaginaceae
Plantago tomentosa Lam.	Plantaginaceae
Polygala linoides Poir.	Polygalaceae
Pteridium aquilinum (L.) Kuhn	Dennstaedtiaceae
Pterocaulon sp.	Asteraceae
Rhynchosia diversifolia Micheli	Fabaceae
Richardia humistrata (Cham. et Schlecht.) Steud.	Rubiaceae
Richardia stellaris (Cham. & Schltdl.) Steud.	Rubiaceae
Salvia procurrens Benth.	Lamiaceae
Schizachyrium microstachyum (Desv. ex Ham.) Roseng.	Poaceae
Schizachyrium spicatum (Spreng.) Herter	Poaceae
Schizachyrium tenerum Nees	Poaceae
Setaria parviflora (Poir.) Kerguélen	Poaceae
Setaria vaginata Spreng.	Poaceae
Sida rhombifolia L.	Malvaceae
Sisyrinchium micranthum Cav.	Iridaceae
Sisyrinchium sellowianum Klatt	Iridaceae
Sisyrinchium sp.	Iridaceae
Soliva sessilis Ruiz et Pavón	Asteraceae
Spermacoce verticillata L.	Rubiaceae
Sporobolus indicus (L.) R.Br.	Poaceae
Staelia thymoides Cham. & Schltd	Rubiaceae
Steinchisma hians (Elliott) Nash.	Poaceae
Stenandrium dulce (Cav.) Nees	Acanthaceae
Stipa filifolia Nees	Poaceae
Stipa setigera J.Presl	Poaceae
Stylosanthes leiocarpa Vogel	Fabaceae
Stylosanthes montevidensis Vogel	Fabaceae
Trachypogon spicatus (L. f.) Kuntze	Poaceae
Vernonia flexuosa Sims	Asteraceae
Vernonia hypochaeris DC.	Asteraceae
Vernonia sp.	Asteraceae
Vulpia bromoides (L.) S.F.Gray	Poaceae
Vulpia sp.	Poaceae

Tabela 1: Valores quantitativos dos atributos de solo coletados em 15 populações de *Herbertia zebrina*.

	C1	C2	PI1	PI2	PCH2	PCH1	DF1	DF22	SBN1	SBN2	CA1	CA2	PO21	PO22	FE	Average	SD
pH	5.6	7.2	5.4	5.1	5.3	4.9	5	5	5	5	5.1	5.2	5.9	6	4.7	5.36	0.625
P	2	4.6	4.1	6.1	4.3	4.4	11	10	6.4	10	6.1	4.3	6.2	7.3	6.8	6.24	2.51
K	76	+400	161	169	183	144	190	195	233	328	112	79	112	196	192	184.66	86.83
Al	0	0	0.2	0.2	0.1	0.2	0.1	0.1	0.2	0.1	0.1	0.1	NA	NA	0.2	0.12	0.072
Ca	2.2	1.9	1.9	4.1	4.6	3.9	7.6	8.2	4.8	5.2	2.3	2.3	2.1	2.2	2.7	3.73	2.04
Mg	1.6	1.3	1.9	2	0.9	1.5	2.9	4	2.3	2.9	1.6	1.7	1.4	1.7	2	1.98	0.78
S	2.4	16	3.7	5.2	2.2	3.8	5.3	12	6.1	9.6	7.8	7.8	3.4	4.9	5.4	6.37	3.78
Zn	1	1	1.8	5	1.3	2	6.6	7.2	1.7	6.9	3.2	4.6	3.5	3	3.8	3.50	2.14
Cu	0.2	0.2	0.8	0.3	0.1	0.5	1.4	1.4	0.5	1.3	1.3	1.2	0.4	0.4	0.8	0.72	0.48
B	0.2	0.2	0.3	0.5	0.4	0.4	0.4	0.6	0.7	0.6	0.5	0.6	0.3	0.4	0.4	0.43	0.15
Mn	21	12	36	23	8	19	47	57	26	34	26	24	15	25	27	26.66	12.78
Fe	0.7	0.2	1.9	0.6	1	0.6	3.5	6.3	1.6	1.9	1.7	NA	NA	NA	1	1.75	1.68

Tabela 1: Unidades atributes de solo analisado: pH(H_2O), P(mg/dm^3), Al($cmol_c/dm^3$), Ca($cmol_c/dm^3$), Mg($cmol_c/dm^3$), S(mg/dm^3), Zn(mg/dm^3), Cu(mg/dm^3), B(mg/dm^3), Mn(mg/dm^3), Fe(g/dm^3) and Na(mg/dm^3).

Fotos do experimento de germinação de sementes realizado em 2016

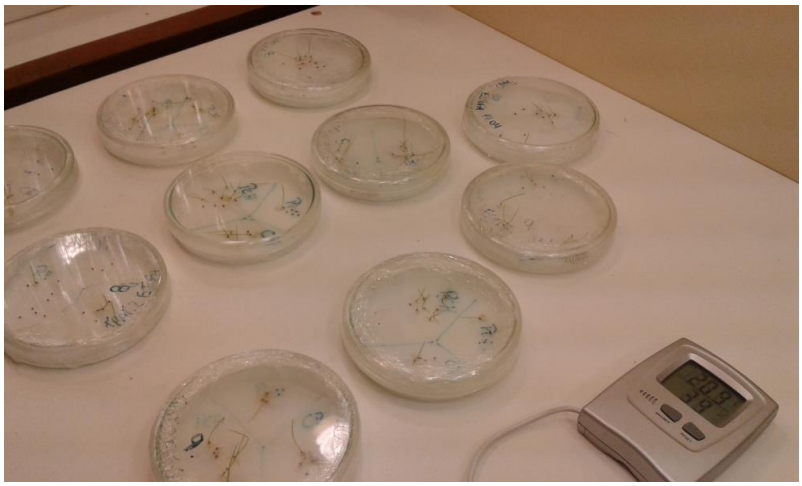

Figura 1: Foto mostrando as placas com semente de *H. zebrina* situadas na câmera de germinação onde o experimento foi realizado.

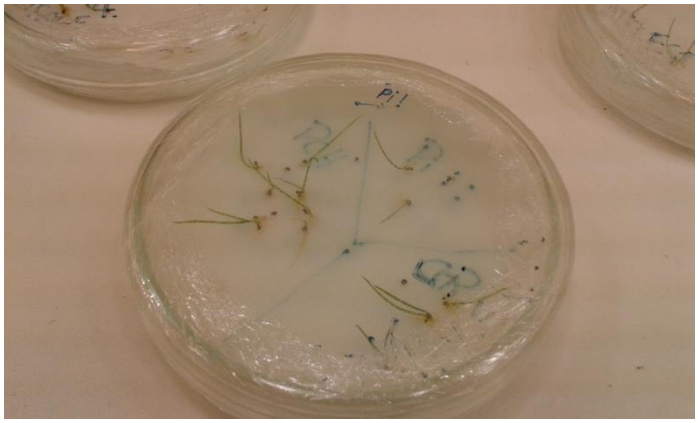

Figura 2: Foto mostrando em ampliação uma das placas de germinação e o aparecimento de folhas em várias das sementes de *H. zebrina*.

Figura 3: Foto mostrando o processo de germinação de sementes de *H. zebrina*. Na imagem é possível observar a emissão da radícula e também o aparecimento da primeira folha.

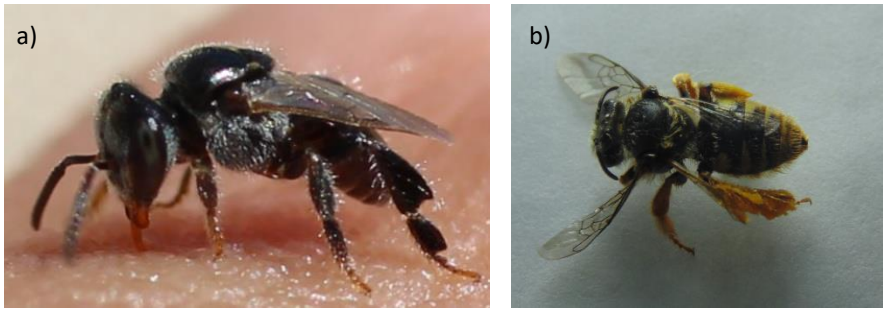

Figura 4: Fotos de dois dos principais polinizadores de *H. zebrina.* a) *Trigona spinipes* que coleta pólen na espécie estuda e *Lanthanomelissa betinae* uma das espécies identificadas como coletora de óleo.