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Aspectos evolutivos e da diversidade genética das espécies de *Sisyrinchium* L. pertencentes ao clado V.

Juliana Maria Fachinetto

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Orientadora: Prof[a]. Dr[a] Tatiana Teixeira de Souza Chies

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Dedico à minha família.

Ando devagar porque já tive pressa
E levo esse sorriso porque já chorei demais
Hoje me sinto mais forte mais feliz, quem sabe
Só levo a certeza de que muito pouco sei
Ou nada sei
....
Penso que cumprir a vida seja simplesmente
Compreender a marcha e ir tocando em frente.

(Almir Sater)

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RESUMO

Sisyrinchium (Sisyrinchieae: Iridoideae: Iridaceae) é o maior gênero em número de espécies de Iridaceae. Este gênero ocorre em todo o continente americano, com o centro de origem e distribuição na América do Sul. Aproximadamente 35% das espécies de *Sisyrinchium* possuem flores com elaióforos que podem produzir óleos lipídicos como recompensa aos polinizadores. Suas espécies apresentam ampla diversidade morfológica intra e inter-populacional tornando a taxonomia de *Sisyrinchium* extremamente complicada. Classificações morfológicas dividem o gênero em oito seções, no entanto, recentes estudos filogenéticos identificaram nove clados que pouco se assemelham com as classificações taxonômicas prévias. Dentre os clados de *Sisyrinchium,* é importante destacar o clado V, formado por pelo menos duas seções: *Lenitium* e *Scirpeocaris*. Todas as espécies pertencentes a este clado possuem tricomas produtores de óleos florais (elaióforos) localizados na coluna estaminal.

Visando contribuir para o conhecimento da biologia e evolução das espécies de *Sisyrinchium*, este estudo teve por objetivo investigar a evolução e características genéticas e citogenéticas de diferentes espécies de *Sisyrinchium* pertencentes ao clado V, além de aspectos da biologia reprodutiva para as duas categorias morfológicas de *S. sellowianum*. Dados de ISSR (*Inter Simple Sequence Repeats*) foram obtidos para diferentes espécies do clado V de *Sisyrinchium* para acessar a variação genética, estrutura populacional e fluxo gênico entre espécies. Dados citogenéticos foram utilizados para determinar o número cromossômico destas espécies. Separadamente, *S. sellowianum* foi classificada em duas categorias morfológicas devido à variação das populações naturais observadas nesta espécie. Dados de ISSR, número cromossômico, morfologia e viabilidade polínica, além de aspectos reprodutivos foram utilizados para caracterizar as categorias morfológicas de *S. sellowianum* (CM-I e CM-II). Para o estudo de relacionamento filogenético entre as espécies do clado V de *Sisyrinchium* foi amostrado um número representativo de espécies com as características deste clado e amplificadas duas regiões de DNA, ITS (nuDNA) e *trnQ-rps16*(cpDNA).

Do estudo filogenético, 4,37% dos caracteres foram filogeneticamente informativos, sendo a região ITS1 a mais informativa. As análises suportaram a monofilia das espécies *S. commutatum, S. setaceum, S. sellowianum* CM-II e *S. scariosum*. As

demais espécies avaliadas não formaram grupos suportados. Os dados de ISSR indicam que as populações das diferentes espécies de *Sisyrinchium* encontram-se fortemente estruturadas (F_{ST}= θ^B= 0.51) e não apresentam correlação entre distância genética e geográfica. Três níveis de ploidia foram observados entre as espécies deste clado, com populações diploides (2n= 2x= 18), tetraploides (2n= 4x= 36) e hexaploides (2n= 6x= 54), sendo o número básico x = 9. As populações de *S. sellowianum* mostraram alta diferenciação genética (F_{ST} = 0.46, θ^B= 0.62). Em relação à distribuição da variação genética, foi observada maior variação intra-populacional (69%) que inter-populacional (31%) na CM-I, enquanto em CM-II, 61% da variação foi observada entre as populações e 39% intra-populacional. Também não foi observada correlação entre distância genética e geográfica para as populações de *S. sellowianum*. No entanto, uma correlação significativa foi observada entre distância genética e categoria morfológica das populações de *S. sellowianum.* Quanto ao número cromossômico, CM-I apresentou populações diploides (2n= 2x= 18) e tetraploides (2n= 4x= 36), enquanto CM-II apenas populações diploides (2n= 2x= 18). O estudo polínico revelou diferentes morfologias polínicas em cada categoria morfológica, além de correlação entre tamanho dos grãos de pólen e nível de ploidia na CM-I. As categorias morfológicas de *S. sellowianum* também se diferenciam em relação ao modo reprodutivo, CM-I é alógama, enquanto CM-II é autógama, não havendo formação de frutos em cruzamentos realizados entre as categorias morfológicas.

A alta estruturação populacional pode ser explicada pela distância entre as populações, o que possivelmente reduz o fluxo gênico devido ao comportamento dos polinizadores. Os polinizadores descritos para as espécies de *Sisyrinchium* produtoras de óleos florais são abelhas coletoras de óleos da família Apidae, tribo Tapinotaspidini, as quais geralmente forrageiam áreas restritas. As categorias morfológicas de *S. sellowianum* são geneticamente e morfologicamente diferenciadas e refletem seus modos reprodutivos. Os resultados obtidos podem auxiliar na delimitação destas duas categorias morfológicas como duas espécies ou subespécies, o que foi fortemente suportado nas análises filogenéticas. A técnica de ISSR não foi capaz de discriminar as diferentes espécies de *Sisyrinchium,* assim como os dados obtidos de duas sequências de DNA, os quais discriminaram apenas algumas espécies. Estes estudos indicam que dados genéticos sozinhos não são capazes de delimitar as espécies deste clado, tornando necessária a utilização de diferentes técnicas e abordagens para obter dados evolutivos e de

relacionamento filogenético mais acurado, como os gerados para as duas categorias morfológicas de *S. sellowianum*.

ABSTRACT

Sisyrinchium (Sisyrinchieae: Iridoideae: Iridaceae) is the largest in number of species of Iridaceae. This genus occurs throughout the American continent, with the center of origin and distribution in South America. Approximately 35% of *Sisyrinchium* species have flowers with elaiophores that can produce lipid oils as a reward to pollinators. Its species have a wide morphological diversity within and among populations making the taxonomy of *Sisyrinchium* extremely complicated. Morphological classifications proposed eigth sections to genus, however, recent phylogenetic studies identified nine clades do not correspond to the previous taxonomic classifications. Among the clades of *Sisyrinchium*, it is important to highlight the clade V, formed by at least two sections: *Lenitium* and *Scirpeocaris*. All species belonging to this clade possess trichomes producers of floral oils (elaiophores) located in the staminal column.

Aiming to contribute for the understanding of the biology and evolution of *Sisyrinchium* species, this study investigated the evolution and genetic plus cytogenetic traits of different species belonging to the clade V, and aspects of reproductive biology for the two morphological categories of *S. sellowianum*. ISSR data were obtained for the different species of *Sisyrinchium* clade V in order to assess genetic variation, population structure and gene flow among species. Cytogenetic data were used to determine the chromosome number of these species. Separately, *S. sellowianum* was classified into two categories due to morphological variation in natural populations observed in this species. ISSR data, chromosome number, morphology and viability pollen plus reproductive parameters were used to characterize the morphological categories of *S. sellowianum* (MC-I and MC-II). To evidence the phylogenetic relationships among species of *Sisyrinchium* of clade V a representative number of species was sampled with the characteristics of this clade and amplified two DNA regions, ITS (nuDNA) and *trnQ-rps16* (cpDNA).

Concerning the phylogenetic study, 4.37% of the characters were phylogenetically informative, with the ITS1 the most informative. The analysis supported the monophyly of the species *S. commutatum*, *S. setaceum*, *S. sellowianum* MC-II and *S. scariosum*. The other species did not form supported groups. ISSR data indicate that populations of different species of *Sisyrinchium* are strongly structured ($F_{ST}= \theta^B= 0.51$) and no correlation between genetic and geographic distances. Three ploidy levels were

observed among species of this clade, with diploid (2n= 2x= 18), tetraploid (2n= 4x= 36) and hexaploid (2n= 6x= 54) populations, with the basic number x = 9. The populations of *S. sellowianum* showed high genetic differentiation (F_{ST} = 0.46, θ^B= 0.62). Regarding the distribution of genetic variation, greater intra-population variation (69%) that inter-population (31%) was observed in MC-I, while the MC-II, 61% of the variation was observed among populations and 39% intra-population. Also no correlation between genetic and geographic distance was observed for the populations of *S. sellowianum*. However, a significant correlation between genetic distance and morphological category of the populations of *S. sellowianum* was observed. Regarding the chromosome number, MC-I showed diploid (2n= 2x= 18) and tetraploid (2n= 4x= 36) populations, while MC-II only diploid (2n= 2x= 18) populations. The pollen study revealed different pollen morphologies in each morphological category, and correlation between pollen pollen size and ploidy level in MC-I. Morphological categories of *S. sellowianum* also differ in relation to the reproductive mode, MC-I is alogamous, while MC-II is autogamous, with no fruit development in crosses made between morphological categories.

The high population structure can be explained by distance among populations, which possibly reduces gene flow due to the behavior of pollinators. Pollinators described for the species of *Sisyrinchium* producing floral oils are oil-bees of the family Apidae, tribe Tapinotaspidini, which generally restricted foraging areas. Morphological categories of *S. sellowianum* are genetically and morphologically differentiated and reflect their mating systems. The results can assist in the delimitation of two morphological categories such as two species or subspecies, which was supported highly in phylogenetic analysis. The ISSR technique was not able to discriminate different species of *Sisyrinchium,* as well as the data obtained by the two DNA sequences, which discriminate only a few species. These studies suggest that genetic data alone are not able to delimit species of this clade, making necessary the use of different techniques and approaches to obtain evolutionary and phylogenetic relationship more accurate, as those generated for the two morphological categories of *S. sellowianum*.

CAPÍTULO I

INTRODUÇÃO GERAL

O gênero *Sisyrinchium* apresenta uma grande diversidade de formas, além de grande variabilidade intra e inter-específica e populacional. Fotos: Lilian Eggers.

1. A Família Iridaceae

Iridaceae está entre as maiores famílias da ordem Asparagales, com cerca de 2030 espécies distribuídas entre 65-75 gêneros (Goldblatt *et al*., 2008; APG III, 2009). Possui ampla distribuição mundial, resultado do intenso processo de radiação adaptativa em Iridaceae, com dois centros de diversidade: continente africano (37 gêneros e cerca de 1190 espécies) e sul-americano (24 gêneros e mais de 210 espécies) (Goldblatt e Manning, 2006; Goldblatt *et al*., 2008; Goldblatt e Manning, 2008).

Esta família possui representantes de grande importância econômica. Gêneros como *Crocus* L., *Dietes* Salisbury ex Klatt, *Freesia* Eckl. ex Klatt, *Gladiolus* L., *Iris* L., *Moraea* Mill e *Watsonia* Mill., são especialmente cultivados visando a indústria de flores de corte e para paisagismo devido a beleza de suas espécies e cultivares (Goldblatt *et al*., 1998; Goldblatt e Manning, 2008). Na culinária, *Crocus sativus* L. é conhecido pela produção do açafrão, um condimento muito apreciado, de coloração vermelha, obtido a partir dos seus estiletes e estigmas (Hosseinzadeh *et al*., 2005). Em regiões da Síria e da África, os cormos de várias espécies são consumidos como alimento (Goldblatt *et al*., 1998; Goldblatt e Manning, 2008).

1.1 Aspectos Morfológicos de Iridaceae

As espécies de Iridaceae podem ser plantas decíduas ou perenes, herbáceas em sua maioria, exceto pelos gêneros *Klattia* Baker, *Nivenia* Vent. e *Witsenia* Thunb. que possuem hábito arbustivo (Goldblatt *et al*., 1998).

Considerando o aspecto vegetativo, Iridaceae reúne plantas que apresentam raízes adventícias típicas de monocotiledôneas, órgãos subterrâneos do tipo rizomas, cormo ou bulbo (Goldblatt *et al*., 1998). As folhas são simples, equitantes ou alternas dísticas dispostas ao longo do caule ou na base da planta, podendo ser cilíndricas ou planas, às vezes invaginantes, de venação paralelinérvea (Judd *et al*., 2009).

As flores são dispostas em inflorescências determinadas monocásio, do tipo ripídio, frequentemente modificadas e às vezes reduzidas a uma flor solitária terminal. São protegidas por brácteas (espatas). Flores perfeitas possuem simetria radial ou bilateral. Apresentam seis tépalas, as externas às vezes diferenciadas das internas, podendo ser livres ou cognatas, imbricadas e vistosas. Os estames são em número de três, opostos às tépalas externas, com filetes livres ou cognatos. O gineceu apresenta três carpelos cognatos e o

ovário é, na maioria das espécies, ínfero (exceto em *Isophysis* Moore), com placentação axial. Os ramos do estilete podem ser expandidos e petaloides, com dois ou três estigmas terminais. O fruto é uma cápsula loculicida, podendo apresentar sementes ariladas ou com uma testa carnosa (Goldblatt, 1990; Goldblatt *et al*., 1998; Goldblatt e Manning, 2008; Judd *et al*., 2009).

As fragrâncias florais são derivadas de uma diversidade de classes biossintéticas, incluindo derivados de ácidos graxos, compostos benzênicos e isoprenoides; os odores florais podem ser muito variados entre espécies e, em certos casos, dentro de espécies (Goldblatt e Manning, 2006).

As recompensas florais são do tipo néctar, pólen ou óleos florais não voláteis. O néctar é o mais comum, produzido em nectários septais localizados dentro das paredes radiais do ovário e em nectários perigoniais (Goldblatt, 1990; Goldblatt e Manning, 2006). Os óleos florais não voláteis são encontrados nas espécies do Novo Mundo e são produzidos em tricomas glandulares chamados elaióforos (Goldblatt, 1990; Goldblatt e Manning, 2006; Renner e Schaefer, 2010; Chauveau *et al*., 2011, 2012). Flores que oferecem óleos como recompensa são encontradas em apenas onze famílias e possuem relação altamente especializada com fêmeas de duas famílias de Hymenoptera, Mellitidae e Apidae (Renner e Schaefer, 2010; Chauveau *et al*., 2012). Essas abelhas possuem modificações morfológicas para obtenção dos óleos florais, que associados ao néctar ou não, são utilizados para alimentação das larvas e, em algumas espécies, como um tipo de selador à prova de água nas paredes das células que compõem o ninho (Renner e Schaefer, 2010).

1.2 Filogenia de Iridaceae

A análise filogenética de Iridaceae que formou a base para a classificação da família utilizou um conjunto de 52 caracteres morfológicos, fitoquímicos, citológicos, anatômicos, embriológicos e de estrutura polínica. De acordo com esta análise cladística, foi possível identificar quatro clados principais (Goldblatt, 1990). Estes clados receberam a classificação de subfamília e foram denominados Isophysidoideae (monogenérico, *Isophysis*), Nivenioideae, Iridoideae e Ixioideae (Goldblatt, 1991).

Em uma análise cladística subsequente de Iridaceae, Rudall (1994) usou 33 caracteres anatômicos e morfológicos, com maior ênfase nas evidências anatômicas. Este

estudo chegou a resultados um pouco diferentes, onde os gêneros de Crocoideae (anteriormente denominada Ixioideae) foram considerados grupo irmão para o restante da família, a qual ficou dividida em dois grupos, um correspondente à subfamília Iridoideae e outro Nivenioideae mais o gênero *Isophysis*.

Devido à dificuldade em se obter uma classificação de consenso entre algumas subfamílias de Iridaceae, alguns estudos posteriores utilizando sequências de DNA foram realizados visando esclarecer essas dúvidas. Souza-Chies *et al*., (1997) realizaram o primeiro estudo para a família, utilizando o gene de cloroplasto *rps4*. Os autores demonstraram que a família Iridaceae tem origem monofilética e suportou a posição de *Isophysis* como membro de Isophysidoideae e como um grupo irmão para o restante da família.

Posteriormente, Reeves *et al*. (2001) utilizando quatro regiões de DNA plastidial (os genes codificadores *rbcL*, *rps4*, o íntron *trnL* e o espaçador intergênico *trnL-F*) obtiveram resultados congruentes aos observados por Goldblatt (1990) e Souza-Chies *et al*., (1997).

Atualmente, a classificação aceita para Iridaceae foi obtida a partir de um estudo filogenético multigênico, realizado por Goldblatt *et al*. (2008), utilizando o íntron *rps16*, a região *trnL-F* e os genes *rbcL*, *rps4* e *matK.* Foi proposta a divisão em sete subfamílias: Isophysidoideae, Patersonioideae, Geosiridoideae, Aristeoideae, Nivenioideae, Crocoideae e Iridoideae. A partir deste estudo realizado por Goldblatt *et al* (2008), foi inferida, através de relógio molecular, uma estimativa de tempo de divergência entre o ancestral de Iridaceae e a família Doryanthaceae. A divergência entre Iridaceae e Doryanthaceae ocorreu há cerca de 82 milhões de anos, e o gênero *Isophysis* (Isophysidoideae) considerado o mais remoto da família, principalmente pela presença de flores com ovário súpero, teria divergido das Iridaceae ancestrais há cerca de 66 milhões de anos.

No Novo Mundo, estão presentes apenas representantes da subfamília Iridoideae, a qual é monofilética (Goldblatt, 1990, Reeves *et al*., 2001; Goldblatt *et al*., 2008). A subfamília Iridoideae compreende cinco tribos: Tigridieae, Trimezieae, Sisyrinchieae, Irideae e Diplarreneae (Goldblatt *et al*., 2008).

1.3 Citogenética de Iridaceae

Iridaceae apresenta número cromossômico básico, tamanho cromossômico e

morfologia cariotípica muito variáveis, o que torna a análise citológica uma importante ferramenta para esclarecer a sistemática e a evolução desta família (Goldblatt e Takei, 1997).

A poliploidização é considerada um dos processos mais marcantes na evolução das Angiospermas, de maneira que eventos de duplicação podem ser rastreados em 70-100% das espécies diploides atuais (Masterson, 1994; Simillion *et al.*, 2002; Blanc e Wolfe, 2004; Leitch e Leitch, 2008; Soltis *et al.*, 2009; Jiao *et al.*, 2011).

Em Iridaceae, a poliploidização é um fator marcante na evolução da família. Foi proposta para a família uma origem paleopoliploide após um evento ancestral de duplicação do genoma. Cerca de 60% das espécies de Iridaceae no Hemisfério Norte são neopoliploides (poliploidia intragenérica). No continente africano, cerca de 5% das espécies de Crocoideae e 10% de Iridoideae são neopoliploides. No entanto, nas Américas Central e do Sul, a poliploidia parece ter sido mais importante na evolução da família, onde muitas das espécies de Tigridieae são tetra ou hexaploides e em Sisyrinchieae, mais de 75% das espécies de *Sisyrinchium* são poliploides (Goldblatt e Takei, 1997). De acordo com Kenton e Heywood (1984), nas espécies de Iridaceae sul-americanas ocorre um número considerável de espécies poliploides e estes poliploides possuem menores níveis de ploidia do que as espécies do Hemisfério Norte.

Diversos estudos citológicos têm demonstrado que além da poliploidia, a familia é bastante diversa em termos de cariótipo, número cromossômico básico e conteúdo de DNA (Goldblatt, 1982; Goldblatt *et al.*, 1984; Kenton e Heywood, 1984; Goldblatt e Takei, 1997). Importante para a evolução, outros diferentes processos citológicos, como aneuploidia, disploidia e alterações estruturais cromossômicas exercem importantes papéis no surgimento de novas espécies (Sharma e Talukdar, 1960). Em Iridoideae, outro aspecto particularmente comum é a bimodalidade cariotípica (Kenton *et al.,* 1990; Goldblatt e Takei, 1997). Variação no tamanho dos cromossomos e no conteúdo de DNA também vem sendo observados (Souza-Chies *et al.,* 2012), além de variações numéricas intra e interespecíficas (Alves *et al.*, 2011).

O número básico de cromossomos para Iridaceae ainda é incerto. Possivelmente, x= 10 seja o número básico ancestral para a família (Goldblatt, 1990; Goldblatt *et al.*, 1998; Goldblatt e Takei, 1997). Provavelmente para as subfamílias Nivenioideae, Iridoideae e Crocoideae o número básico seja x= 10, a julgar pela

distribuição dos números básicos dentro dos gêneros menos especializados (Goldblatt, 1990). No entanto, ocorrem muitos números derivados, tais como x= 9, 8, 7 e 5, e essa variação pode resultar em descendentes disploides. De acordo com Alves *et al*. (2011), o número cromossômico (2n) em Iridaceae varia de 6 a aproximadamente 230, reforçando a poliploidia como um evento importante na evolução das espécies desta família. Alguns gêneros americanos são exclusivamente poliploides, como *Fosteria* Molseed, *Cobana* Ravenna e *Sessilanthera* Molseed & Cruden, com número básico x= 7 (Goldblatt *et al*., 1998).

Na subfamília Tigridieae, o gênero *Herbertia* possui número cromossômico básico x= 7 e parece não apresentar alterações. Seis das sete espécies reconhecidas têm contagens cromossômicas inferidas e quatro níveis de ploidia são relatados: *2x*, *4x*, *6x* e *8x* (Winge, 1959; Kenton e Heywood, 1984; Goldblatt e Takei, 1997; Roitman e Castilho, 2004; Moreno *et al*., 2009). Para *Calydorea*, as espécies estudadas possuem número básico x= 7 e três níveis de ploidia, 2x, 4x e 6x (Goldblatt, 1982; Goldblatt e Takei, 1997).

Dentro da subfamília Iridoideae é verificada uma ampla diversidade quanto à morfologia e tamanho cromossômico. Em *Sisyrinchium*, os números cromossômicos são altamente variáveis, devido à poliploidia e a possíveis eventos de hibridação natural; para grande parte das espécies os cromossomos são geralmente pequenos (2 – 4 µm) (Henderson, 1976; Goldblatt et al., 1982; Cholewa e Henderson, 1984; Kenton *et al*., 1986; Rudall *et al*., 1986).

Diferentes números cromossômicos básicos ocorrem em *Sisyrinchium* (*x* = 5, 6, 8, 9, 17). Os mais frequentes são x=8, principalmente nas espécies da América do Norte (Kenton e Heywood, 1984) e x=9 na América do Sul (Souza-Chies *et al*., 2012). Goldblatt (1982) sugeriu que x= 9 seria o número cromossômico básico ancestral para o gênero e x= 8 o mais derivado. Esta hipótese foi sustentada pela reconstrução filogenética de *Sisyrinchium*, já que as espécies com número básico x= 9 são pertencentes aos clados que divergiram mais precocemente do ancestral comum (Chauveau *et al*., 2011).

O nível de ploidia tem sido relacionado a diversos fatores. Alguns estudos reportam uma correlação positiva entre nível de ploidia e latitude para espécies de Iridaceae do Hemisfério Norte, onde plantas com altos níveis de ploidia (6x - 12x) ocorrem em latitudes maiores (Goldblatt, 1982; Kenton *et al*., 1986; Rudall *et al*., 1986).

Estudos realizados em espécies de *Sisyrinchium* do Hemisfério Norte indicam

uma relação entre sistema de cruzamento e nível de ploidia (Ingram, 1967; Henderson, 1976). Estes estudos mostram que a autofertilização é frequente nos dodecaploides, enquanto os tetraploides são auto-incompatíveis.

1.4 Aspectos gerais da biologia reprodutiva em Iridaceae

Iridaceae é uma interessante família de plantas do ponto de vista de biologia reprodutiva. A diversidade de estruturas florais das espécies desta família atrai uma gama de diferentes visitantes florais, como abelhas, besouros, moscas de probóscide longa e curta, borboletas, mariposas e pássaros (Benhardt e Goldblatt, 1998; Goldblatt e Manning, 2006).

As espécies subsaarianas de Iridaceae têm sido bem estudadas por diversos autores (Goldblatt *et al*., 1989; Goldblatt *et al*., 1995; Johnson 1996; Goldblatt, 1997; Steiner, 1998; Goldblatt e Manning, 2000; Johnson *et al*., 2003; Manning, 2004; Goldblatt e Manning, 2006; Anderson *et al*., 2009), no entanto, sistemas de polinização de Iridaceae fora da África têm recebido pouca atenção (Cocucci e Vogel, 2001; Freitas e Sazima, 2003; Devoto e Medan, 2004; Sapir *et al*., 2005).

Muitos gêneros de Iridaceae nativas do sul da África têm uma relação positiva entre diversidade de espécies e diversidade de sistemas de polinização (Bernhardt e Goldblatt, 2000). Significativamente, a diversidade de sistemas de polinização aumenta primariamente com a complexidade floral e secundariamente com o tamanho do gênero (Goldblatt e Manning, 2006).

O gênero *Sparaxis* Ker Gawl, um pequeno gênero endêmico da África, apresenta três sistemas de polinização sobrepostos, que exploram três ordens de insetos como vetores de pólen (Coleoptera, Diptera e Hymenoptera) (Goldblatt *et al*., 2000). Já o gênero *Romulea* Maratti, com cerca de 90 espécies, nas 32 espécies avaliadas, que incluem todos os tipos florais, a polinização é realizada por uma faixa relativamente estreita de insetos (Goldblatt *et al*., 2002). *Hesperantha* Ker Gawl, outro gênero de Iridaceae, apresenta quatro sistemas de polinização sobrepostos que exploram insetos de quatro ordens (Coleoptera, Diptera, Hymenoptera e Lepidoptera) (Goldblatt *et al*., 2004).

Como esperado em sistemas de polinização predominantemente por especialistas, atrativos e recompensas florais são bem relacionados com o perfil do polinizador, resultando no desenvolvimento de distintas síndromes florais (Faegri e Van de Pijl, 1979).

Deste modo, 95% das espécies são polinizadas por uma ou poucas espécies de insetos e outros animais, enquanto apenas 3% são visitadas por uma gama maior de polinizadores (Goldblatt e Manning, 2008).

Os atrativos são principalmente pigmentação do perianto complementado por odor floral em muitas espécies, mas a forma da flor, orientação das tépalas e simetria floral, podem ser igualmente importantes para alguns polinizadores. A recompensa para visitantes na maioria das espécies de Iridaceae é néctar, mas em outras é pólen e óleos não voláteis (Goldblatt e Manning, 2006).

Flores que oferecem lipídeos florais como recompensa foram registradas, até o momento, em apenas 11 famílias de Angiospermas (Renner e Schaeffer, 2010). Estima-se que este caráter tenha evoluído independentemente 28 vezes (Renner e Schaeffer, 2010). Para a maioria das famílias, o surgimento de glândulas secretoras de lipídeos, possivelmente tenha ocorrido uma única vez na história evolutiva dessas famílias, exceto em Orchidaceae e Iridaceae, que apresentam múltiplas origens (Goldblatt *et al.*, 2008; Chase *et al.*, 2009; Renner e Schaeffer, 2010).

Em Iridaceae, os óleos florais constituem o terceiro tipo de recurso ofertado para polinizadores, juntamente com pólen e néctar, e são encontrados principalmente em Tigridieae, Trimezieae e Sisyrinchieae (Goldblatt e Manning, 2008; Chauveau *et al.*, 2012). Esse recurso está localizado em tricomas glandulares presentes na coluna estaminal e/ou na face adaxial das tépalas nas espécies de *Sisyrinchium* (Chauveau *et al.*, 2011) e, em Tigridieae e Trimezieae, na face adaxial das tépalas internas na ampla maioria das espécies já estudadas para este caráter (Chauveau *et al.*, 2012).

Em *Sisyrinchium*, 35% das espécies possuem flores com estruturas secretoras de lipídeos, os elaióforos (Chauveau *et al.*, 2011). Neste gênero, os lipídeos secretados são coletados essencialmente por fêmeas de algumas espécies de abelhas da tribo Tapinotaspidini (Hymenoptera: Apidae) equipadas com estruturas especializadas na coleta de lipídeos florais (Cocucci e Vogel, 2001; Alves-dos-Santos *et al.*, 2007). Esses tricomas glandulares podem ter exercido um importante papel na diversificação de *Sisyrinchium* (Chauveau *et al.*, 2011).

O modo de reprodução (sexual ou assexual), assim como o sistema de cruzamento (auto ou alogamia), estão diretamente relacionados com a composição genética de populações vegetais, pois o primeiro determina se há ou não formação de gametas, e o

segundo, define os padrões pelos quais os gametas são reunidos (Bodanese-Zanettini e Cavalli, 2003).

Ambos os modos de reprodução estão presentes em Iridaceae. A formação de bulbos é comum em espécies da tribo Tigridieae e em algumas espécies de *Iris* (Iridoideae), e a formação de cormos em espécies de Crocoideae e Iridoideae (Goldblatt e Manning, 2008).

A reprodução sexual pode ser por autogamia (autofecundação), alogamia (fecundação cruzada) e misto (autógamo e alógamo simultaneamente) (Karasawa, 2009). O principal sistema de cruzamento nas espécies de Iridaceae é a fecundação cruzada, facilitada pela autoincompatibilidade e hercogamia (Bernhardt e Goldblatt, 2000). No entanto, as espécies de Iridaceae também podem ser autocompatíveis, ocorrendo, desta maneira, alogamia, autogamia e sistema misto de cruzamento (Goldblatt e Manning, 2008).

Em *Sisyrinchium*, os sistemas de cruzamento variam de completa autoincompatibilidade a completa autocompatibilidade (Ingram, 1967; Henderson, 1976; Cholewa e Henderson, 1984). Além disso, existem estudos, realizados com várias espécies do gênero, que mencionam a formação de plântulas férteis a partir do eixo de duas brácteas florais, caracterizando, dessa forma, reprodução vegetativa (Cholewa e Henderson, 1984).

É proposta uma associação entre sistema de cruzamento e nível de ploidia para as espécies de *Sisyrinchium* do Hemisfério Norte, sendo frequente autofertilização em dodecaploides, enquanto os tetraploides são autoincompatíveis (Ingram, 1967; Henderson, 1976). Outros estudos sugerem essa mesma associação, onde a autocompatibilidade e autofecundação ocorrem em táxons com altos níveis de ploidia (superior a 4x), já os tetraploides tendem a ser autoincompatíveis ou realizam alogamia mesmo sendo autocompatíveis (Henderson, 1976; Cholewa e Henderson, 1984).

Truylio *et al*. (2002) descreveram *S. micranthum* como uma espécie auto-incompatível. Entretanto, Tacuatiá *et al*. (2012a) encontrou uma forte relação entre o nível de ploidia e o sistema de cruzamento, onde as populações diploides e tetraploides de *S. micranthum* são auto-incompatíveis, mas as hexaploides são autocompatíveis.

Henderson (1976) também verificou em espécies de *Sisyrinchium* do Hemisfério Norte, que conforme o nível de ploidia, o grau de expressão da protandria mostrava-se alterado, tendo efeitos diretos no sistema de cruzamento. Neste caso, quanto maior o nível de ploidia da espécie investigada, menos pronunciado era o intervalo de tempo entre a

maturação das anteras e do estigma.

Em *Sisyrinchium bermudiana* L., já foi descrita uma variação no comprimento do filete (Kenton *et al*., 1986). Os autores observaram que quando a antera apresenta o mesmo comprimento do estilete existe uma maior probabilidade de ocorrer autopolinização.

1.5 Iridaceae no Brasil

No Brasil, encontram-se espécies nativas e exóticas da família Iridaceae. Ocorrem 19 gêneros (três deles endêmicos), distribuídos em 165 espécies, das quais 70 são exclusivas do Brasil (Eggers *et al.,* 2013). As espécies estão distribuídas desde a caatinga e a região do cerrado, ao longo da Floresta Atlântica, até a região Sul do país, nos campos e na Floresta de Araucárias (Eggers, 2008).

Espécies nativas da família ainda estão escassamente estudadas devido ao curto período de florescimento, à fragilidade das flores e à dificuldade de preservação das características morfológicas importantes para definição das espécies (Eggers, 2008).

Os gêneros mais estudados encontram-se na região sudeste, destacando-se *Neomarica* Sprague, *Trimezia* Salisb. ex Herbert e *Pseudotrimezia* R. C. Foster (Chukr, 1992a; 1992b; Chukr e Giulietti, 2001). Em termos de diversidade e abundância, os gêneros mais representativos são *Sisyrinchium* L. (58 espécies), *Neomarica* (21 espécies), *Pseudotrimezia* (21 espécies), *Trimezia* (14 espécies) e *Cypella* Herb. (14 espécies) (Eggers *et al*., 2010).

No Rio Grande do Sul, as espécies nativas de Iridaceae destacam-se durante o florescimento na primavera. Foram até o momento, identificados doze gêneros, com 59 espécies: *Sisyrinchium* (tribo Sisyrinchieae), *Trimezia* e *Neomarica* (tribo Trimezieae) e *Calydorea* Herbert, *Catila amabilis* Ravenna, *Cypella*, *Gelasine* Herbert, *Kelissa brasiliensis* Ravenna, *Onira unguiculata* Ravenna, *Phalocallis coelestis* (Lehm.) Ravenna e *Herbertia* Sweet (tribo Tigridieae) (Eggers *et al.,* 2013) (Fig. 1). Algumas espécies são endêmicas e acredita-se que outras ainda não sejam conhecidas no meio botânico.

2. O Gênero *Sisyrinchium*

Sisyrinchium, pertence à subfamília Iridoideae, tribo Sisyrinchieae (Goldblatt e Manning, 2008). É o maior gênero em número de espécies de Iridaceae no Novo Mundo e compreende cerca de 140 espécies (Goldblatt e Manning, 2008). Baseado em caracteres

morfológicos, *Sisyrinchium* está dividido em oito seções: *Sisyrinchium, Echtronema, Spathirhachis, Lenitium, Scirpeocaris, Hydastylus, Segetia* e *Viperella* (Ravenna, 2000, 2001a, 2001b, 2002, 2003a, 2003b).

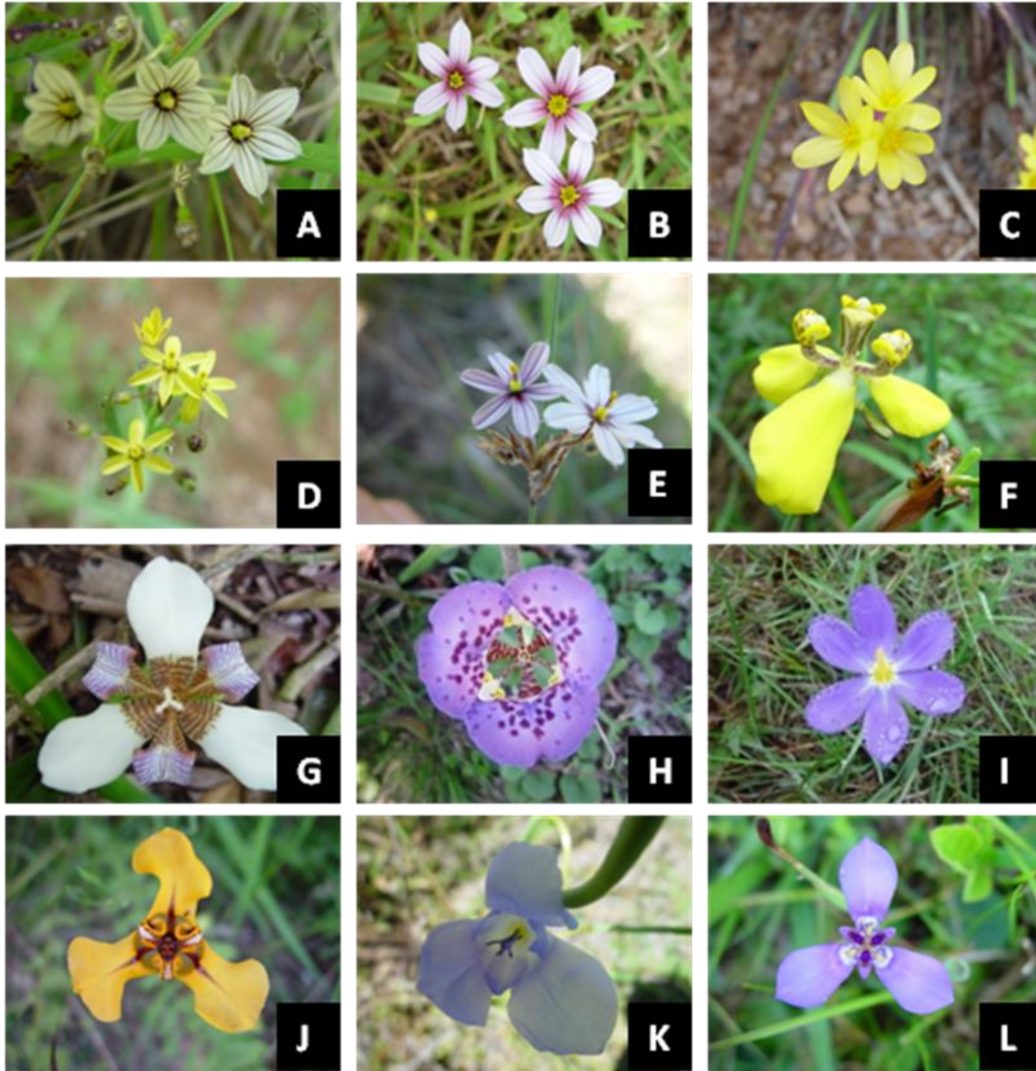


Figura 1 – Espécies de Iridaceae ocorrentes no Rio Grande do Sul. (A) *Sisyrinchium sellowianum*, (B) *Sisyrinchium micranthum*, (C) *Sisyrinchium palmifolium*, (D) *Sisyrinchium commutatum*, (E) *Sisyrinchium scariosum*, (F) *Trimezia* sp., (G) *Neomarica candida*, (H) *Kelissa brasiliensis*, (I) *Calydorea crocoides*, (J) *Cypela herberti*, (K) *Gelasine* sp., (L) *Herbertia lahue*. Fotos: Lilian Eggers.

Sisyrinchium ocorre em todo o continente americano, desde a Groelândia e norte do Canadá até a Terra Do Fogo e Ilhas Falkland (Rudall *et al*., 1986). Johnston (1938) enfatizou a América do Sul como o centro de origem e distribuição de *Sisyrinchium*. Esta proposta está sendo sustentada por diversos estudos (Goldblatt *et al*., 1990; Cocucci e

Vogel, 2001; Goldblatt *et al*., 2008; Chauveau *et al*., 2011), onde o gênero provavelmente tenha surgido entre a Bolívia e o sudoeste dos EUA (Chauveau *et al*., 2011).

Esse gênero compreende espécies de pequeno a médio porte, perenes ou anuais. As raízes são geralmente densas e grossas, na forma de tubérculos, ou fibrosas. As folhas são lanceoladas a lineares ou do tipo terete. As flores são actinomorfas, de coloração variada como creme, amarelo, violáceo e também azul com o centro amarelo e tépalas livres. Os filetes dos estames podem ser parcialmente conectados ou completamente fusionados formando uma coluna estaminal, podendo apresentar tricomas de diferentes tipos (Goldblatt e Manning, 2008; Chukr e Capellari Jr., 2003). Aproximadamente 35% das espécies de *Sisyrinchium* têm flores com elaióforos do tipo tricoma glandular (Chauveau *et al*., 2011). Estes tricomas podem produzir óleos lipídicos como recompensa aos polinizadores (Cocucci e Vogel, 2001).

Sisyrinchium compreende um grupo diverso de espécies que apresentam ampla diversidade morfológica. A taxonomia deste gênero é extremamente complexa (Cholewa e Henderson, 1984; Kenton *et al*., 1986; Rudall *et al*., 1986). Alguns dos caracteres usados pelos botânicos para delimitar as espécies em *Sisyrinchium* são extremamente variáveis, como largura e comprimento das folhas, cor das flores. A fragilidade das flores torna difícil a preservação do material em exsicatas, e a grande variação intra e inter-populacional de diversas características externas exigem que uma grande amostra de indivíduos seja necessária para identificar e descrever as espécies (Henderson, 1976).

Entre as espécies de *Sisyrinchium* que apresentam ampla diversidade morfológica, pode-se citar *S. micranthum* Cav. *Sisyrinchium micranthum* apresenta grande variação no porte dos indivíduos (Chuckr e Cappellari Jr., 2003), o que foi também observado por Tacuatiá *et al*. (2012a,b).

Devido à importância e complexidade de *Sisyrinchium*, este gênero vem sendo objeto de diversos estudos, como análise de caracteres morfológicos, anatômicos, citogenéticos, biologia reprodutiva, além de abordagens filogenéticas e de variabilidade genética. Um estudo envolvendo genética de populações de *S. micranthum*, realizado por Tacuatiá *et al*. (2012a) evidenciou forte diferenciação genética entre as populações analisadas.

Em um recente trabalho utilizando análises filogenéticas baseadas em oito sequências de DNA de regiões do genoma plastidial, mitocondrial e nuclear, Chauveau *et*

al. (2011) trabalharam com 85 espécies de *Sisyrinchium*, num total de 101 acessos. Estes autores confirmaram a monofilia do gênero e demonstraram a formação de nove clados, os quais pouco se assemelham com as classificações taxonômicas obtidas até o momento.

2.1 O Clado V de *Sisyrinchium*

De acordo com Chauveau *et al*. (2011), o gênero *Sisyrinchium* é formado por nove clados. Dentre eles, o clado V é formado por espécies pertencentes a duas seções: *Lenitium* e *Scirpeocaris* (Ravenna, 2000, 2001a, 2001b, 2002, 2003a, 2003b) (Fig. 2).

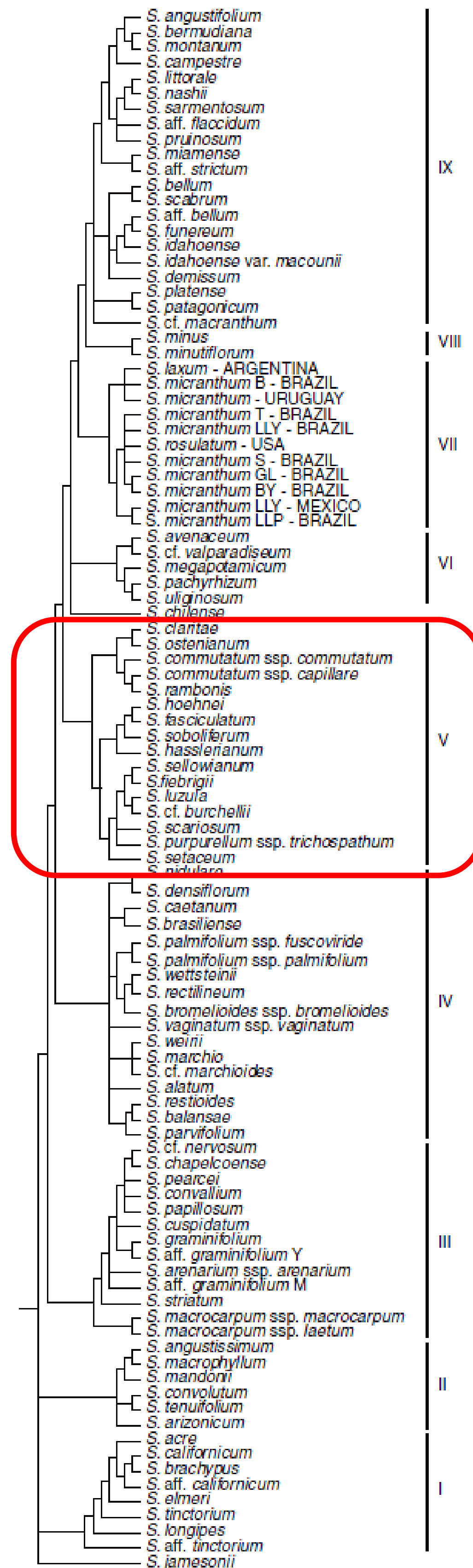
As espécies pertencentes a esse clado apresentam as tépalas largamente planas. Em relação às folhas, estas podem ser cilíndricas ou planas e as flores possuem coloração amarela, róseas ou brancas. Todas as espécies deste clado possuem tricomas produtores de óleos e encontram-se localizados na coluna estaminal (Chauveau *et al*., 2011).

Dentre as espécies pertencentes a este clado estão: *S. claritae* Herter, *S. ostenianum, S. commutatum, S. rambonis* R.C.Foster, *S. sellowianum* Klatt, *S. fiebrigii, S. luzula, S. burchelli, S. scariosum* Johnst, *S. purpurellum, S. setaceum, S. hoehnei, S. fasciculatum, S. soboliferum, S. hasslerianum* Bak. (Fig. 3).

Neste grupo de espécies de *Sisyrinchium*, assim como em outros grupos, há uma grande diversidade intra-específica e inter-populacional, o que dificulta a delimitação das espécies. Os caracteres morfológicos utilizados para determinação dessas espécies apresentam ampla variação, que aliado à carência de estudos, prejudica a taxonomia dessas espécies. Pouca atenção tem sido dada até o momento para esse clado, restringindo-se a coletas e identificações aproximadas das espécies. As espécies pertencentes a esse clado são facilmente identificadas pela morfologia floral, no entanto, definir a espécie a qual pertence determinada população ou indivíduo coletado é bastante desafiador. Dentro desse grupo, diversas populações têm sido identificadas como categorias morfológicas, espécies afins (aff.) e até mesmo há a possibilidade de espécies novas, para as quais não foram encontradas características na literatura que possam incluí-las na descrição de uma espécie já existente.

Esse clado carece de estudos citogenéticos, moleculares, de biologia reprodutiva, e até mesmo morfológicos. Até o momento, na literatura, as espécies pertencentes a esse clado possuem apenas a descrição da espécie e oito sequências de DNA utilizadas para a obtenção da filogenia do gênero *Sisyrinchium* por Chauveau *et al*. (2011).

Figura 2 – Posição filogenética das espécies pertencentes ao clado V de *Sisyrinchium* na árvore filogenética consenso do gênero *Sisyrinchium* (de acordo com Chauveau *et al.*, 2011).



A filogenia de Karst e Wilson (2012) não utilizou nenhuma espécie desse grupo. Devido a essa problemática, torna-se interessante e de extrema necessidade a realização de estudos com essas espécies. A busca de características que possam auxiliar na identificação e na compreensão de tal diversidade pode criar alguns padrões, que podem ser úteis também no entendimento da taxonomia do gênero como um todo.

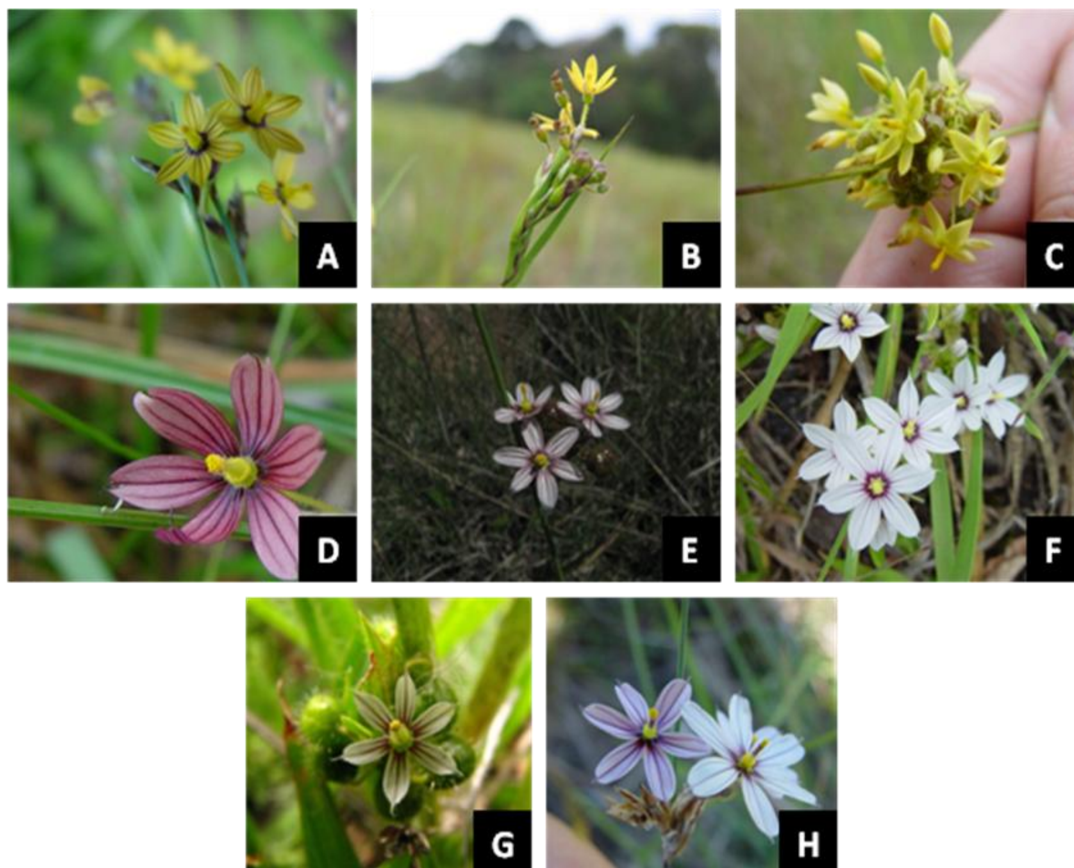


Figura 3 – Espécies de *Sisyrinchium* pertencentes ao clado V (Chauveau *et al*., 2011): (A) *S. claritae*, (B) *S. commutatum*, (C) *S. hoehneii*, (D) *S. luzula*, (E) *S. purpurellum* subsp. *purpurellum*, (F) *S. sellowianum* CM-I, (G) *S. sellowianum* CM-II, (H) *S. scariosum*. Fotos: Lilian Eggers.

3. Estudos de diversidade genética baseados em marcadores moleculares de DNA

A variabilidade genética, além de importante para a evolução, pode ser utilizada como instrumento de investigação por ecólogos e sistematas em diversos ramos como, por exemplo, na verificação das afinidades e os limites entre as espécies, e detecção sobre os modos de reprodução e estrutura populacional, para estimar níveis de migração e dispersão nas populações (Avise, 1994).

Entre as principais características que influenciam a variabilidade genética em plantas está a distribuição geográfica das populações que compõem determinada espécie e o sistema de reprodução: as espécies consideradas como endogâmicas têm os maiores valores de variabilidade genética entre as categorias de distribuição geográfica (Hamrick *et al.*, 1979). A distribuição geográfica tem um papel importante não apenas nas variantes que determinam a adaptação ecológica de uma espécie, mas também em características que podem resultar em processos de especiação. Quanto mais distanciadas se encontram as populações que compõem uma determinada espécie, as suas frequências alélicas e as características de base genética tendem à maior variação, uma particularidade provavelmente relacionada à variedade de condições ecológicas que são impostas para a sua adaptação (Futuyma, 2002).

Marcadores moleculares de DNA têm sido utilizados com sucesso em estudos de diversidade genética e biologia evolutiva. Entre os métodos mais utilizados em plantas, encontram-se os marcadores microssatélites ou polimorfismo de sequências simples repetidas (SSR), o polimorfismo de comprimento de fragmentos amplificados (AFLP), o polimorfismo de DNA amplificado ao acaso (RAPD) e, mais recentemente, os marcadores baseados na variação de intersequências simples repetidas (ISSR).

Os ISSR são marcadores arbitrários multiloci produzidos por amplificação por PCR (Reação em Cadeia da Polimerase) com *primers* (inicializadores) correspondentes a microssatélites. Os ISSR apresentam abundante polimorfismo, não requerem conhecimento genômico prévio, possuem alta reprodutibilidade e, além disso, são de custo relativamente baixo (Zietkiewicz *et al.*, 1994; Bornet e Branchard, 2001). A tecnologia de marcadores baseados em ISSR foi desenvolvida a partir de microssatélites e explora a abundância e a distribuição aleatória das sequências simples repetidas ao longo dos genomas eucarióticos, amplificando as sequências de DNA contidas entre os microssatélites (Rossato *et al.*, 2007; Brandão, 2008).

Os marcadores ISSR têm herança dominante, ou seja, apenas um alelo por loco é detectado, todos os outros alelos são agrupados em uma classe simbolizada pela ausência da banda no gel de eletroforese. Devido ao seu comportamento dominante, esses marcadores fornecem limitada informação por loco quando comparados aos marcadores co-dominantes (Han *et al.*, 2009). Uma alta reprodutibilidade pode ser obtida com a escolha de temperaturas de anelamento adequadas à sequência dos *primers* (Bornet e

Branchard, 2001). Algumas medidas podem ser úteis para melhorar a qualidade da interpretação do sistema de bandas e elas incluem o uso de controles positivos nas eletroforeses (Bonin *et al.*, 2007) além da utilização de amostras em replicatas (Bornet e Branchard, 2001; Bonin *et al.*, 2007).

Em Iridaceae, marcadores moleculares têm sido utilizados com sucesso tanto para a caracterização molecular de cultivares e de espécies (Caiola *et al.*, 2004; Marco *et al.*, 2009; Moraga *et al.*, 2010; Ranjan *et al.*, 2010; Tacuatiá *et al.*, 2012a), quanto para a obtenção de estimativas de parâmetros genéticos intra e interpopulacionais (Hannan e Orick, 2000; Wróblewska *et al.*, 2003; Tacuatiá *et al.*, 2012a).

As questões evolutivas envolvidas no processo de especiação em *Gladiolus* puderam ser melhor entendidas por meio de um estudo onde foi correlacionado parâmetros de genética populacional obtidos por marcadores AFLP com análise espacial e de fenologia (Rymer *et al.*, 2010).

Levando em conta que o gênero *Sisyrinchium* apresenta uma grande variabilidade e problemas na delimitação de espécies, a combinação de estudos morfológicos e moleculares é fundamental para o entendimento da taxonomia desse grupo. Um estudo realizado por Spier *et al.* (2008) revelou que o uso de marcadores moleculares do tipo PCR-RFLP é eficiente para estimar a variabilidade genética de alguns gêneros estudados de Iridaceae e para confirmar a identificação de suas espécies. Além do mais, estes mesmos autores demonstraram que as espécies estudadas agruparam-se conforme a tribo, Sisyrinchieae ou Tigridieae, e dentro da tribo Sisyrinchieae, as espécies foram agrupadas de acordo com a presença ou ausência de elaióforos.

Atualmente, várias espécies da família Iridaceae com ocorrência no sul do Brasil vêm sendo investigadas em termos de diversidade genética e genética de populações utilizando marcadores do tipo ISSR (Souza-Chies *et al.*, 2012). Esses estudos podem contribuir de forma significativa quanto ao conhecimento das espécies endêmicas ou de ocorrência restrita ao sul da América do Sul, pois representam estudos pioneiros na investigação da distribuição da variabilidade genética no continente sul-americano, o qual representa um dos grandes centros de diversificação de espécies da família Iridaceae (Goldblatt *et al.*, 2008).

Além da importância na utilização dos marcadores recém descritos, cabe ressaltar o uso de sequenciamento de fragmentos específicos de DNA, que permite detectar

qualquer mutação de ponto, e tem sido utilizado em estudos de sistemática e filogenia molecular. Em sistemática vegetal ao nível intragenérico, destacam-se fragmentos codificados pelo DNA plastidial (cpDNA) e os espaçadores ITS rDNA do genoma nuclear. Até agora, poucos estudos têm sido realizados utilizando marcadores moleculares nucleares para investigar a diversidade de espécies de Iridaceae (Wróblevska *et al.*, 2003; Caiola *et al.*, 2004; Meerow *et al.*, 2005; Meerow *et al.*, 2007; Tacuatiá *et al.*, 2012a).

Em *Sisyrinchium,* é importante destacar os dois mais recentes estudos filogenéticos realizados para o gênero. Chauveau *et al.* (2011) utilizando 101 acessos representando 85 espécies de *Sisyrinchium* e oito sequências de DNA do genoma plastidial, mitocondrial e nuclear confirmaram a monofilia para o gênero sugerindo a subdivisão de *Sisyrinchium* em nove clados principais. Os autores também investigaram a evolução dos tricomas produtores de óleos, e sugerem que a presença destes tricomas evoluiu, ao menos, três vezes independentemente em *Sisyrinchium* além de terem desempenhado um papel fundamental na diversificação do gênero. Além disso, os padrões biogeográficos indicam expansões das Américas Central e Norte para os Andes entre Chile e Argentina e para a bacia do Rio Paraná. Karst e Wilson (2012) utilizaram 75 acessos num total de 60 espécies de *Sisyrinchium* e dois marcadores de DNA (ITS e *mat*K/*trn*K). Estes autores também confirmaram a monofilia para o gênero, no entanto sugerem a subdivisão em oito clados principais, baseados na amostragem utilizada.

Os estudos filogenéticos em *Sisyrinchium* sugerem novos agrupamentos os quais pouco se assemelham com as classificações taxonômicas utilizadas até o momento (Chauveau *et al.*, 2011; Karst e Wilson, 2012). As relações filogenéticas destes novos agrupamentos propostos merecem ser investigadas para um melhor entendimento dos processos evolutivos envolvidos com as diferentes espécies de *Sisyrinchium.*

OBJETIVO GERAL

O presente estudo está inserido em um amplo projeto que visa contribuir com o conhecimento da biologia e evolução das espécies de *Sisyrinchium.* Diante da complexidade biológica e taxonômica do gênero *Sisyrinchium*, esta tese objetiva reunir informações que possam auxiliar no entendimento de questões evolutivas e da diversidade

genética associadas a variações morfológicas das espécies de *Sisyrinchium* pertencentes ao clado V.

OBJETIVOS ESPECÍFICOS

- Analisar o relacionamento filogenético das espécies pertencentes ao clado V de *Sisyrinchium* através de dados de sequências de DNA e auxiliar na delimitação das espécies deste grupo.

- Avaliar a diversidade intra e interpopulacional através da técnica de ISSR-PCR de populações de onze espécies pertencentes ao clado V de *Sisyrinchium*;

- Determinar o nível de ploidia de onze espécies de *Sisyrinchium* pertencentes ao clado V;

- Avaliar, comparativamente, a diversidade intra e interpopulacional através da técnica de ISSR-PCR de diferentes populações das duas categorias morfológicas de *S. sellowianum*;

- Analisar comparativamente as duas categorias morfológicas de *S. sellowianum* quanto ao sistema reprodutivo, nível de ploidia, morfologia polínica, tamanho e viabilidade dos grãos de pólen;

CAPÍTULO II

An updated phylogeny for *Sisyrinchium* (Sisyrinchieae: Iridaceae) species belonging to clade V.

As espécies de *Sisyrinchium* pertencentes ao clado V apresentam também uma grande variabilidade no porte dos indivíduos.
Fotos: Olivier Chauveau e Lilian Eggers.

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FACHINETTO, CHAUVEAU, EGGERS, KALTCHUK-SANTOS, SOUZA-CHIES
PHYLOGENY OF THE CLADE V OF SISYRINCHIUM.

An updated phylogeny for *Sisyrinchium* (Sisyrinchieae: Iridaceae) species belonging to clade V.

Juliana Fachinetto,[1] Olivier Chauveau,[2] Lilian Eggers,[2] Eliane Kaltchuk-Santos,[1] Tatiana Teixeira de Souza-Chies.[1,2,3]

[1]Programa de Pós-Graduação em Genética e Biologia Molecular da Universidade Federal do Rio Grande do Sul, CEP 91501-970, Porto Alegre, Rio Grande do Sul, Brasil

[2]Departamento de Botânica, Universidade Federal do Rio Grande do Sul, CEP 91501-970, Porto Alegre, Rio Grande do Sul, Brasil

[3]Author for Correspondence (tatiana.chies@ufrgs.br)

***Abstract* –** *Sisyrinchium* is an important genus of Iridaceae, originated in the Americas. In a recent phylogenetic study, this genus was divided into nine well resolved clades. Some species have a such remarkable morphological variation that make difficult its circumscription. In this study, the relationships among the species of the clade V was analyzed. 60 accessions distributed in 17 species and five possible new species were used to amplify two DNA regions, ITS (nuDNA) and *trnQ-rps16* (cpDNA). From molecular datasets, Maximum Parsimony, Maximum likelihood and Bayesian analyses were performed. A total of 1878 characters were obtained of which 4.37% were phylogenetically informative. The analyses demonstrated that the species of the clade V correspond to a single natural group, although belonging to two morphological sections, *Lenituium* and *Scirpeocaris*. The species *S. commutatum*, *S. setaceum*, *S. sellowianum* MCII (morphological category II) and *S. scariosum* grouped with moderately to strong support. This study is in agreement with other, using ISSR markers, focused in this clade, where the populations did not grouped according to species, except for *S. sellowianum* MCI (morphological category I). In conclusion, the ITS region was the most informative to investigate the relationships of *Sisyrinchium* species and other species of evolutionary complex lineages. The twelve groups formed showed several synapomorphic characters which may have resulted in support to the groups. Furthermore, some accessions of *Sisyrinchium* formed supported groups, as *S. sellowianum* MC-II, reinforcing the possibility of a new species.
***Keywords* –** *Lenitium,* phylogenetic analyses, *Scirpeocaris*, synapomorphy.

INTRODUCTION

Iridaceae is an important family of Monocots, encompassing approximately 2000 species distributed among 65 to 75 genera (Goldblatt and Manning 2008). South America is one of the main centers of species diversification in Iridaceae (Goldblatt et al. 2008). Of the seven subfamilies of Iridaceae (Goldblatt et al. 2008), Iridoideae is the major evolutionary branch of the family, comprising at least 900 species and making up about 40% of the species richness within the Iridaceae (Goldblatt and Manning 2008). Iridoideae is divided into five tribes and the major dichotomy is the separation of the Old World genera (Diplarreneae and Irideae) and the New World genera (Sisyrinchieae, Trimezieae and Tigridieae) (Goldblatt et al. 2008).

Sisyrinchium L. is a large and complex genus of Sisyrinchieae, comprising about 140 species (Goldblatt et al. 2008; Goldblatt and Manning 2008). This genus is distributed in the American continents from subarctic areas to Tierra del Fuego (Chauveau et al. 2011). The taxonomy of this genus is extremely complex (Cholewa and Henderson 1984; Kenton et al. 1986; Rudall et al. 1986).

Several species of Iridaceae, including *Sisyrinchium* species, occurring in Southern Brazil have been investigated in terms of genetic diversity and population genetics using ISSR markers (Souza-Chies et al. 2012; Tacuatiá et al. 2012). Such studies contributed significantly to the knowledge of those species that are endemic or restricted to Southern South America, and represent pioneering studies regarding to the distribution of genetic variability in the South American continent.

Based on morphological characters, *Sisyrinchium* is divided into eight sections: *Sisyrinchium, Echtronema, Spathirhachis, Lenitium, Scirpeocaris, Hydastylus, Segetia* and *Viperella* (Ravenna 2000, 2002a, 2002b, 2003a, 2003b).

Due to the great morphological variability and a complex taxonomy, the genus was recently investigated using molecular markers (Chauveau et al. 2011; Karst and Wilson 2012). The work of Chauveau et al. (2011) combining eight molecular markers of plastidial, mitochondrial and nuclear genomes to 85 species of *Sisyrinchium*, represents the most comprehensive study of the genus. These authors confirm the monophyly of *Sisyrinchium* and revealed nine major clades which have weak connection to subdivisions established by Ravenna (Ravenna 2000, 2002a, 2002b, 2003a, 2003b), with some exceptions.

In particular, the clade V (Chauveau et al. 2011), object of the present study, encompasses species belonging to at least two mofphological sections: *Scirpeocaris* and *Lenitium* (Ravenna 2000, 2002a, 2002b, 2003a, 2003b). The species belonging to this clade are easily identified by the floral morphology, which presented largely flat tepals, the leaves can be cylindrical or flat and all species possess trichomes in the filamental column (Chauveau et al. 2011). However, to identify the species to which belong an individual collected is quite challenging.

According to Chauveau et al. (2011), all members of this clade possess nuptial trichomes on the filamental column and oil secretion. Theses trichomes were densely tufted along the lower third of the column and sparsely distributed on the upper two-thirds. The trichome stalk was long and slender and the blister size varied along the column: trichomes on the lower third exhibiting a larger blister than those on the upper two-thirds. Only *S. fasciculatum*, *S. hasslerianum* and *S. hoehnei* had reflexed trichomes densely tufted along the upper two-thirds of the filamental column and sparsely distributed on the lower third. Already, *S. rambonis* had few trichomes present only at the base of the column.

An important question observed in the clade V is the biological diversity observed in *S. sellowianum*. This species was separated according to morphology of two morphological categories (MC). The MCI has flowers, display floral and floral longevity larger and there is no contact between anther and stigma, while MCII has flowers, display floral and floral longevity smaller and there is an intimate contact between anther and stigma.

According to Karst and Wilson (2012), the circumscription of species in *Sisyrinchium* has been problematic because some species have a wide distribution, some species of groups are morphologically similar, and collections of Central and South American species are generally restricted to regional herbaria. Considering the great variability and problems in the delimitation of species, molecular studies are essential to understand the taxonomy of this group. The use of sequence-specific DNA fragments which can detect any point mutation has been used in studies of systematic and molecular phylogeny.

The goal of this study was to elucidate the phylogenetic relationships among *Sisyrinchium* species belonging to clade V (Chauveau et al. 2011) using a large sampling of species. In addition, this study aims to contribute to the identification of new species within this clade, and verifying the molecular characters able to explain the existing morphological diversity in *S. sellowianum* (MC-I and MC-II).

MATERIAL AND METHODS

Sampling

A total of 60 accessions of *Sisyrinchium*, representing 17 species and five possible new species belonging to the clade V of *Sisyrinchium* (Chauveau et al. 2011) were sampled. These five possible new species are based on morphological characters that distinguish the species already described. Each accession representing a terminal, one individual for population and more than one population was sampled by species (Figure 1; Table S1) when it was possible. *Sisyrinchium palmifolium* (clade IV) and *S. micranthum* (clade VII) (Chauveau et al. 2011) were selected as outgroup.

DNA extraction, amplification and sequencing

Total DNA from fresh or silica gel-dried leaves was extracted using the protocol of Doyle & Doyle (1997) with modifications. A combination of two DNA regions were used to infer phylogenetic relationships among the taxa sampled. The intergenic spacer region *trnQ-rps16* of the plastid DNA and the nuclear ribosomal DNA internal transcribed spacer (ITS) region, including ITS1, ITS2 and the 5.8S gene, were used (Table S2). These sequences were selected according to the parsimoniously informative characters detected in a previous study (Chauveau et al. 2011). PCR amplifications were performed in 30 µL total volume reaction with the following reactions components: 1.5 µL of genomic DNA (approx. 15-50 ng), 1mM of each primer, 250 mM of dNTP, 1x rTaq buffer, 2.5 mM $MgCl_2$ and 0.2 U Taq DNA polymerase (Invitrogen, São Paulo, SP, Brazil) in a termal cycler Veriti 96 Well Thermal Cycle, Applied Biosystems. The PCR conditions for ITS were initial denaturation at 94°C for 5 mn followed by 40 cycles of 94 °C denaturation for 1 mn, 58 °C annealing for 1 mn and 72°C elongation for 1 mn, followed by a final elongation step of 5 mn at 72 °C. The PCR conditions for *trnQ-rps16* were initial denaturation at 94°C for 5 mn followed by 40 cycles of 94 °C denaturation for 30s, 62 °C annealing for 40s and 72°C elongation for 1 mn 30s, followed by a final elongation step of 5 mn at 72 °C. PCR products were purified by enzymatic reaction (*Exonuclease* and *Shrimp Alcaline Fosfatase*) and sequenced using an automatic sequencer ABI PRISM. Raw forward and reverse sequences for each sample were assembled with CodonCode Aligner 3.5.3 (CodonCode Corporation, Dedham, MA, USA); ambiguous bases were corrected after examination of chromatograms, and consensus sequences were edited.

Phylogenetic analyses

Alignments were first produced using MUSCLE (Edgar, 2004) and further improved manually using MEGA5 (Tamura et al. 2011). The indels detected by alignment shared by two or more taxa were coded as binary characters using GapCoder (Young and Healy 2003). The two DNA regions were first independently analyzed (results not shown). Since the comparison of the resulting topologies of the two regions revealed no instances of well-supported topological differences and provided a higher resolution when analyzed together than separately, they were combined. A total-evidence approach was carried out to produce molecular phylogenetic trees.

Datasets were analyzed using the parsimony criterion in PAUP* version 4.0b10 (Swofford 2002). All the Maximum Parsimony (MP) analyses used heuristic searches with 1000 random addition replicates, tree bisection-reconnection (TBR) branches swapping. Consistence and retention index, strict and majority-rule consensus trees were calculated from all most parsimonious trees. The robustness of nodes was evaluated using MP with 1000 bootstrap replicates of new heuristic searches.

To perform the probabilistic analyses, data were partitioned and then the appropriate evolutionary model estimated for each partition by MrModeltest 2.3 (Nylander 2004). The Akaike information criterion was chosen to select the most appropriate model of DNA substitution for each dataset or data partition used in the analyses. Maximum likelihood (ML) analyses were performed using RAxML 7.2.6 (Stamatakis 2006). The confidence of the inferred ML topologies was assessed by nonparametric bootstrap analysis using 1000 pseudo-replicates. MrBayes 3.1.2 (Ronquist and Huelsenbeck 2003) was used to perform the Bayesian analyses using as parameters the calculated evolutionary models. In ML analyses, the evolutionary models used were GTR+I+G and GTR+I to the ITS and *trnQ-rps16* regions, respectively. In Bayesian analyses, the evolutionary models used were GTR+G (ITS1 and ITS2) and K80 (5.8S) to ITS region, and to the *trnQ-rps16* region the model GTR+I.

The groupings inferred from the analyses (MP, ML and Bayesian) were reported on the best tree score. For each analysis, a given value was considered as MP bootstrap (BMP) support or ML (BML) $\geq$ 50%, or if the posterior probability (PP) > 0.90. Strongly supported clusters were defined by BMP and BML $\geq$ 80% and PP $\geq$ 0.95.

Sequence alignments from each region were traced to assess differentiation at the molecular level and to compare molecular diversity with patterns of morphological

similarity among populations in the groups supported. In addition, the characters of all accessions of *S. sellowianum* (MC-I and MC-II) were analyzed, in comparison, searching for characters that could differentiate the two morphological categories.

RESULTS

The ITS sequence matrix presented an aligned length of 691 characters, including ITS1, 5.8S rRNA gene and ITS2. Of these characters, 102 were variable sites and 56 parsimony informative (PI) across 62 samples (Table 1). The general frequencies of the four nucleotides A, C, G and T were, respectively, 22.0, 31.2, 30.0 and 16.8, showing high guanine-cytosine (GC) content (61.2%). In this region *indels* were not found. In the maximum parsimony analysis, the heuristic search for ITS data was limited by Maxtrees in 13,781 most parsimonious trees with 125 evolutionary steps with a consistency index (CI) of 0.58 and retention index (RI) of 0.82 (Table 1). The MP, ML and Bayesian topology tree is very similar using the ITS region.

The *trnQ-rps16* sequence matrix presented and aligned length of 1187 characters, including only spacer *trnQ*. Of these characters, 56 were variable sites and 14 parsimony informative (PI) across 62 samples (Table 1). The general frequencies of the four nucleotides A, C, G and T were, respectively, 32.9, 14.0, 15.2 and 38.0, showing high adenine-thymine (AT) content (70.9%). In this region five *indels* were found. In the maximum parsimony analysis, the heuristic search for *trnQ-rps16* data was limited by Maxtrees in 9,562 most parsimonious trees with 117 evolutionary steps with a consistency index (CI) of 0.52 and retention index (RI) of 0.75 (Table 1). The MP, ML and Bayesian topology tree is very similar using the *trnQ-rps16* region.

No incongruence between the topologies resulting from the individual analysis of the two DNA regions was observed. Thus, the molecular datasets was combined for analysis, a total of 1878 characters, of which 82 (4.37%) were phylogenetically informative. The ITS1 region was individually more parsimoniously informative (2.08%).

MP analysis of the two DNA regions combined resulted in 64,554 most parsimonious trees with 151 steps, consistence index (CI) = 0.55, retention index (RI) = 0.79. ML searches produced a best ML tree with –ln L = 4036.8. The Bayesian analysis resulted in 36,593 trees retained, which were summarized in the 50% majority-rule consensus tree.

The MP, ML and Bayesian analysis supported the monophyly of the clade V (BMP=

BML= 88%, PP= 1; with a strong support) (Figure 2). The monophyly of the species *Sisyrinchium commutatum* (group II and III) and *S. setaceum* (group V) were strongly supported (BMP = BML = 88%, PP = 0.99), as well as ESC 361 (*S.* cf. *burchelli*) e ESC 362 (*Sisyrinchium* sp.02) (group X) (BML= 99%) and ESC 363 (*Sisyrinchium*. sp.03) e ESC 246 (*S.* aff. *luzula*) (group XII) (BMP=BML= 80%, PP= 0.99). The three accessions of *S. sellowianum* MCII (group XI) also formed a monophyletic group (BMP = BML = 100%, PP = 0.99), moreover, these accessions are not grouped with accessions of *S. sellowianum* MCI. The three accessions of *S. scariosum* (group IV) formed a monophyletic group with moderate support (BML= 62%). The others species did not form monophyletic supported groups.

Analyzing the supported groups in the phylogenetic trees, the two regions were informative including deletion, insertion, inversions, transversion and transition that can help in the identification of species and clarify the relationship among species (Table S3). These groups showed higher number of transition than transversion for apomorphic characters in two regions. Insertions and deletions were detected in two regions; however, only in *trnQ-rps16* indels were found (Table S3). In *trnQ-rps16*, two deletions, in site 236-249 and 871-878, were found in all groups formed in the phylogenetic tree (Table S3) and in all species belonging to clade V (data not showed).

Analyzing only accessions of *S. sellowianum* (MC-I and MC-II), we observed seven characters occurring in a morphological category and do not occur in the other. All the characters were found in ITS region, with five transitions and two transversions (Table 2). The differences observed in sites 60, 101, 125, 229 and 455 were exclusives to this group (group XI), not being shared by any group formed in the phylogenetic tree.

Discussion

The monophyly of *Sisyrinchium* was confirmed in several studies using morphological data (Goldblatt 1990) and DNA sequences (Goldblatt et al. 2008; Chauveau et al. 2011; Chauveau et al. 2012, Karst and Wilson 2012). Phylogenetic studies in *Sisyrinchium* suggest several main clades within the genus which are only partially congruent with the subdivision recognized previously (Chauveau et al. 2011; Kart and Wilson 2012). This study was based on the phylogeny obtained by Chauveau et al (2011), that include a larger number of species and most representative of all sections described by Ravenna (2000,

2002a, 2002b, 2003a, 2003b) for the genus *Sisyrinchium* than by Karst and Wilson (2012). In this study, molecular evidences were used to infer phylogenetic relationships among species of *Sisyrinchium* belonging to clade V. Monophyly of this clade was confirmed. Although the species of this clade belong to two different morphological sections, *Lenitium* and *Scirpeocaris*, our results demonstrate that the two sections do not correspond to two clades. The species analyzed in this study correspond to a single natural group of plants in recent adaptative radiation.

Most of the formed groups correspond to a single species, as can be seen in the groups II, III, IV, V, VI, VII and XI, although some of these species, as *S. hoehneii* formed two supported groups (groups VI and VII).

Resolution and support is low for some species, providing insufficient evidence to determine their monophyly and relationships among them. In agreement with our data, Karst and Wilson (2012) evidenced that several *Sisyrinchium* species, especially among those that are widespread, are not monophyletic.

According to Goldblatt and Takei (1997) 75% of the species of *Sisyrinchium* are polyploidy. For some species of the clade V, variation in ploidy level occur within species, as in *S. luzula*, which present diploids, tetraploids and hexaploids populations (Fachinetto et al. unpublished). In some cases, different cytotypes may be reproductively isolated and may actually represent cryptic species (Soltis et al. 2007), while in other cases substantial gene flow or shared ancestral variation among cytotypes could happened (Ramsey et al. 2008).

Incongruence between ITS and plastid topologies were observed also in species of tribe Trimezieae (Lovo et al. 2012) suggesting evidences of hybridization and introgression cycles, important processes in diversification of Iridaceae (Souza-Chies et al. 2012; Harpek et al. 2013). Although, in this study, no conflicts were observed between topologies inferred from chloroplast (*trnQ-rps16*) vs. nuclear (ITS) sequences among species did not possible exclude reticulate evolution by high similarity of the sequences.

In addition, low phylogenetic resolution in the clade V probably is attributed to the lack of informative DNA sequence variation due to recent divergence within the genus and the number of accessions used by species. In some species, only one accession was used, as in *S.* cf. *burchellii, S. fasciculatum, S. fiebrigii, S. hasslerianum* and *S. rambonis*. According to Whittall et al. (2006) the potential for polymorphism within and among species will

require larger sample sizes.

This species belonging to clade V of *Sisyrinchium* were analyzed by dominant markers (ISSR), and the pattern of groupments among the populations of different species revealed similar results to those obtained here. The populations analyzed also did not grouped according to species and very low support values were obtained (Fachinetto et al. unpublished). In addition, autopoliploidization events was suggest for the species of *Sisyrinchium,* because even those species occurring in sympatry have low values of gene flow (Nm) and differenciated gene pools with low admixture proportions (Fachinetto et al., unpublished).

Reconstruct evolutionary relationships among closely related species using DNA sequences data is often complicated by insufficient data for adequate resolution (Datwyler and Wolf 2004). However, in some species of plants, the nuclear ribosomal internal transcribed spacer (ITS) region and chloroplast intergenic spacers have been used to examine species-level relationships with considerable success (Soltis and Soltis 1998), while among closely related species, these DNA regions are sometimes insufficient to solve relationships (Small et al. 1998). In the present study, the low resolution of the trees can be due to the close relationships among species of the clade V of S*isyrinchium*, although suitable markers have been used.

The supported group in phylogenetic tree showed a large number of synapomorphies, which explains the high values of support of the branch (Table S3). Among the twelve groups formed in the phylogenetic tree, only the group IV (*S. scariosum*) clustered with moderate amounts of support. In such group, the accession SP089 showed several nucleotide differences in comparison with the other two accessions from *S. scariosum*. These differences may have reduced the values of support this group.

The species *S. sellowianum* has been classified into two morphological categories (Fachinetto et al. unpublished). *S. sellowianum* MCI presents largest plants and flowers, largest flower formations and herkogamy when compared to *S. sellowianum* MCII. Furthermore, studies with both morphological categories reveal other differences, such as reproductive system, pollen morphology, occurrence of polyploidy and genetic diversity. In this study, the monophyly has not been confirmed for *S. sellowianum* MCI (Fig. 2).

Associated to morphological diversity concerning *S. sellowianum*, differences in DNA sequences also were found in the ITS region, where five transitions and two transversions

(Table 2). These results corroborate other studies about this species, which revealed differences in population structure, pollen morphology and reproductive system (Fachinetto et al. unpublished). Based on all results to *S. sellowianum*, it is possible to suggest a taxonomic revision for this species.

In this study, the ITS region was the most informative in number of variable characters. A study based on DNA barcode using 98 species of *Sisyrinchium* and related genera (tribe Sisyrinchieae) identified the ITS region as the more appropriate and produced the best results (Alves et al. 2014). However, these authors reported the difficulties of the DNA barcode to indentify species in evolutionary complex lineages. Because of the low number of informative characters in DNA sequences makes is difficult to obtain a good resolution in the phylogenetic tree to infer the relationships among species.

This study represents a preliminary search to investigate the relationships among species within clades of *Sisyrinchium*. A larger number of markers is necessary in order to increase the support of the branches, to assist in the delimitation of species. Integrating morphological, cytological and molecular markers and reproductive data approaches may be more effective in investigating the evolutionary relationships among species of this clade, as well as the description of new species that may exist.

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Figure 1 – Distribution of the accessions collected of the species of *Sisyrinchium*.

Table 1 – Comparison between the DNA regions and combined matrix.

Statistics parameters	ITS	*trnQ-rps16*	Combined matrix
Trees length	125	117	151
Variable sites	102	42	170
PI sites	56	14	82
CI	0.58	0.52	0.55
RI	0.82	0.75	0.79
indels	-	5	5

PI= Parsimony-informative; CI= Consistency Index; RI= Retension Index.

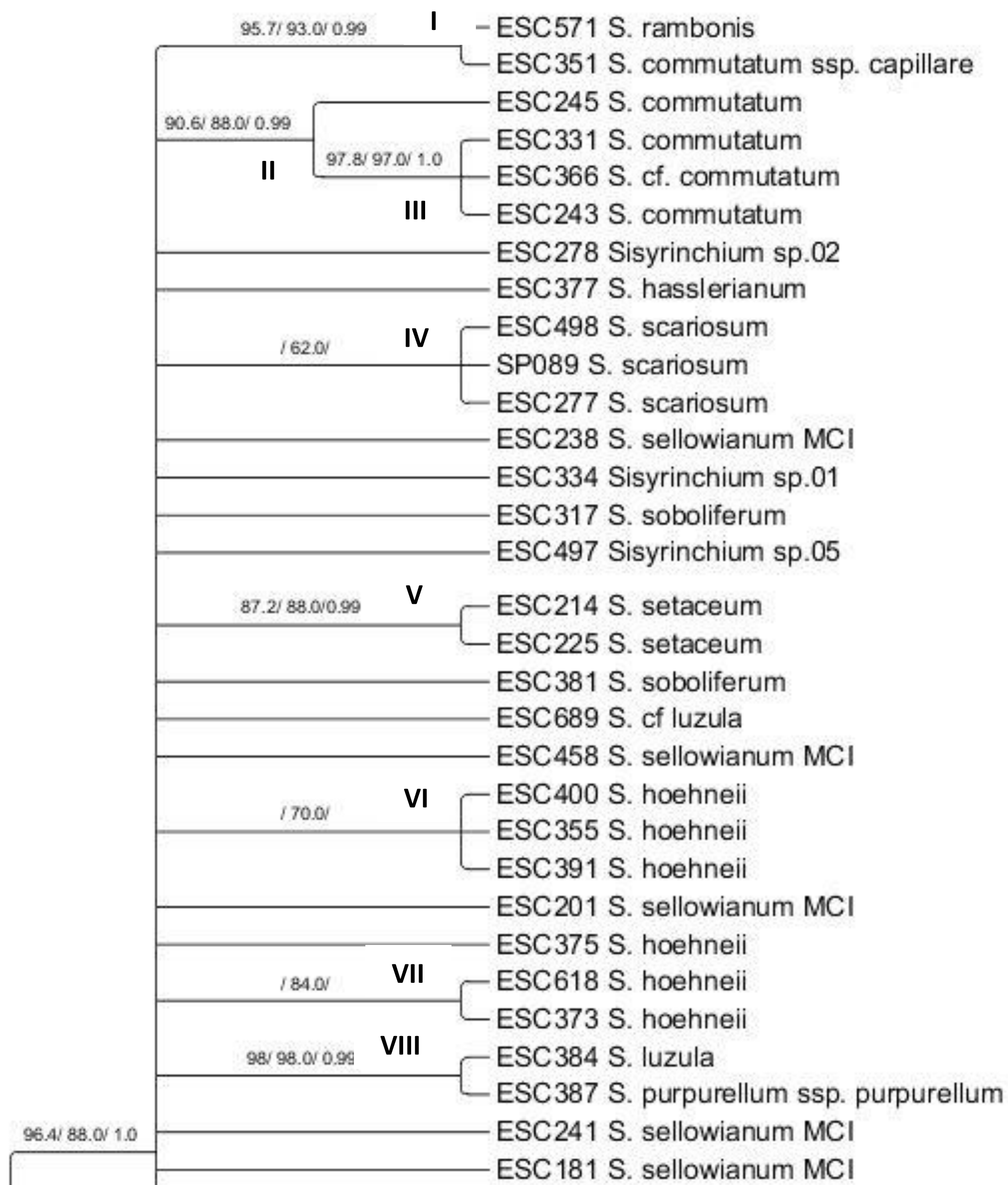
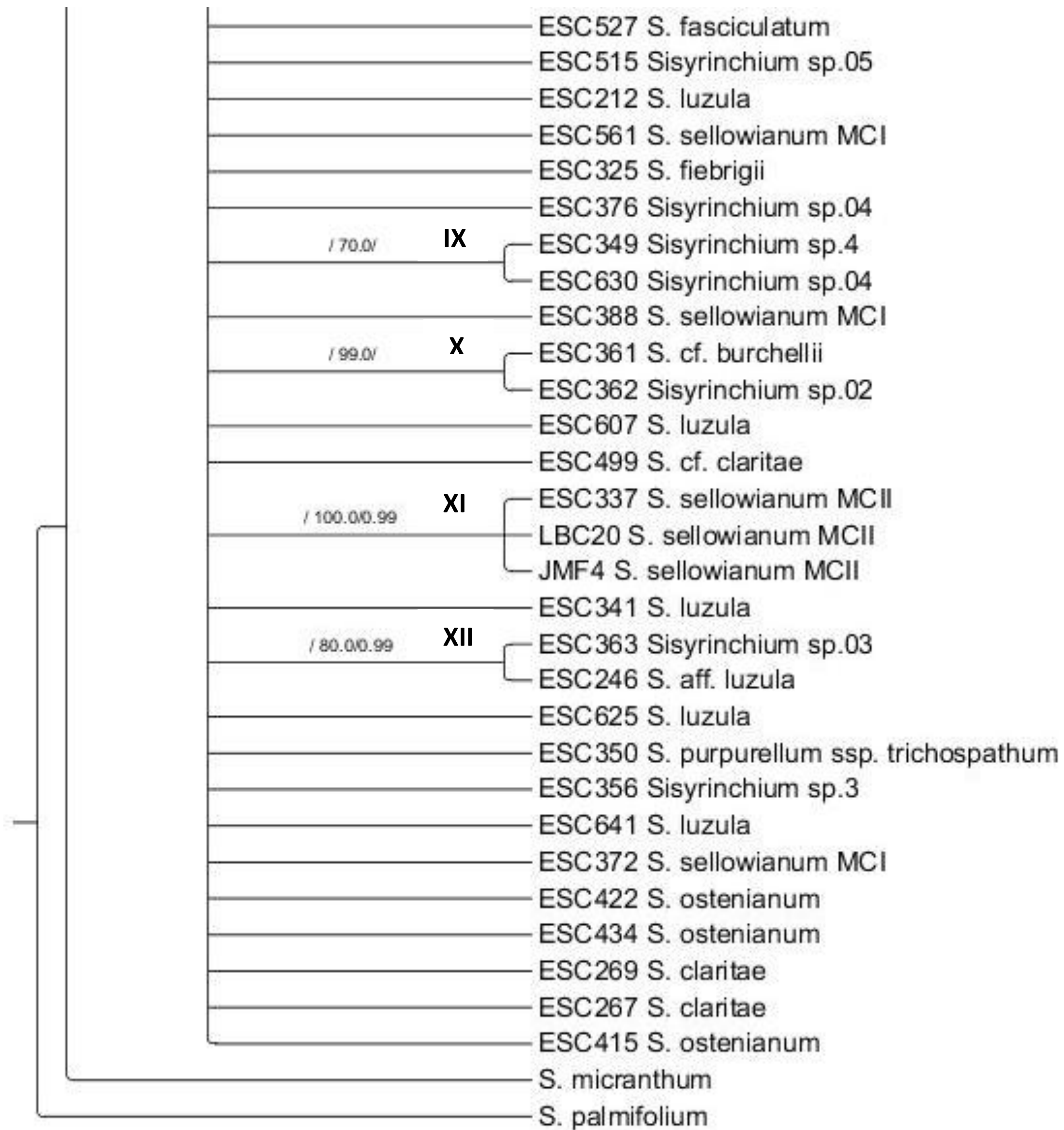


Figure 2 – Maximum-likelihood topology obtained from the full combined molecular dataset. The values above of branch were the support values (BMP/ BML/ PP).



Cont. Figure 2 – Maximum-likelihood topology obtained from the full combined molecular dataset. The values above of branch were the support values (BMP/ BML/ PP).

Table 2 – Polymorphism showed by the two morphological categories in *S. sellowianum*.

	ITS sites						
Morphological Category	60	101	125	182	189	229	455
S. sellowianum MCI	T	A	G	T	T	G	C
S. sellowianum MCII	A	G	A	A	C	A	T

Table S1 – List of taxa sampled of the *Sisyrinchium* to infer the phylogeny in clade V.

Species	Accessions	Section	Localization
S. cf. *burchellii*	ESC361	*Scirpeocaris*	Balsa Nova/PR
S. claritae	ESC267	*Lenitium*	São José do Norte/RS
S. claritae	ESC269	*Lenitium*	São José do Norte/RS
S. cf. *claritae*	ESC499	*Lenitium*	Livramento/RS
S. commutatum	ESC243	*Lenitium*	Teixeira Soares/PR
S. commutatum	ESC245	*Lenitium*	Palmeira/PR
S. commutatum	ESC331	*Lenitium*	Castro/PR
S. cf. *commutatum*	ESC366	*Lenitium*	Palmeira/PR
S. commutatum subsp. *capillare*	ESC351	*Lenitium*	Ponta Grossa/PR
S. fasciculatum	ESC527	*Scirpeocaris*	Barra do Quaraí/RS
S. fiebrigii	ESC325	*Scirpeocaris*	Tijucas do Sul/PR
S. hasslerianum	ESC377	*Scirpeocaris*	Guarapuava/PR
S. hoehneii	ESC355	*Scirpeocaris*	Balsa Nova/PR
S. hoehneii	ESC373	*Scirpeocaris*	Guarapuava/PR
S. hoehneii	ESC375	*Scirpeocaris*	Guarapuava/PR
S. hoehneii	ESC391	*Scirpeocaris*	Palmas/PR
S. hoehneii	ESC400	*Scirpeocaris*	Itaiópolis/SC
S. hoehneii	ESC618	*Scirpeocaris*	Candói/PR
S. luzula	ESC212	*Scirpeocaris*	São Francisco de Paula/RS
S. aff. *Luzula*	ESC246	*Scirpeocaris*	Palmeira/PR
S. luzula	ESC341	*Scirpeocaris*	Jaguariaíva/PR
S. luzula	ESC384	*Scirpeocaris*	Palmas/PR
S. luzula	ESC607	*Scirpeocaris*	General Carneiro/PR
S. luzula	ESC625	*Scirpeocaris*	Castro/PR
S. luzula	ESC641	*Scirpeocaris*	Piraí do Sul
S. cf. *luzula*	ESC689	*Scirpeocaris*	São Joaquim/SC
S. ostenianum	ESC415	*Lenitium*	Viamão/RS
S. ostenianum	ESC422	*Lenitium*	Osório/RS
S. ostenianum	ESC434	*Lenitium*	Caçapava do Sul/RS
S. purpurellum subsp. *trichospathum*	ESC350	*Scirpeocaris*	Ponta Grossa/PR

S. purpurellum subsp. *purpurellum*	ESC387	*Scirpeocaris*	Palmas/PR
S. rambonis	ESC 571	*Sisyrinchium*	Jaquirana/RS
S. scariosum	ESC277	*Scirpeocaris*	São Lourenço do Sul/RS
S. scariosum	ESC498	*Scirpeocaris*	Livramento/RS
S. scariosum	SP089	*Scirpeocaris*	Porto Alegre/RS
S. sellowianum MCI	ESC181	*Lenitium*	Encruzilhada do Sul/RS
S. sellowianum MCI	ESC201	*Lenitium*	São Francisco de Paula/RS
S. sellowianum MCI	ESC238	*Lenitium*	Herciópolis/SC
S. sellowianum MCI	ESC241	*Lenitium*	Pinhão/PR
S. sellowianum MCI	ESC372	*Lenitium*	Guarapuava/PR
S. sellowianum MCI	ESC388	*Lenitium*	Palmas/PR
S. sellowianum MCI	ESC458	*Lenitium*	Tainhas/RS
S. sellowianum MCI	ESC561	*Lenitium*	Caxias do Sul/RS
S. sellowianum MCII	ESC337	*Lenitium*	Sengés/PR
S. sellowianum MCII	JMF4	*Lenitium*	Sapucaia do Sul/RS
S. sellowianum MCII	LBC20	*Lenitium*	Porto Alegre/RS
S. setaceum	ESC214	*Scirpeocaris*	São Francisco de Paula/RS
S. setaceum	ESC225	*Scirpeocaris*	Bom Jardim da Serra/SC
S. soboliferum	ESC317	*Lenitium*	Campo Alegre/SC
S. soboliferum	ESC381	*Lenitium*	Vitorino/PR
Sisyrinchium sp.01	ESC334	-	Jaguariaíva/PR
Sisyrinchium sp.02	ESC278	-	São Lourenço do Sul/RS
Sisyrinchium sp.02	ESC362	-	Balsa Nova/PR
Sisyrinchium sp.03	ESC356	-	Balsa Nova/PR
Sisyrinchium sp.03	ESC363	-	Palmeira/PR
Sisyrinchium sp.04	ESC349	-	Jaguariaíva/PR
Sisyrinchium sp.04	ESC376	-	Guarapuava/PR
Sisyrinchium sp.04	ESC630	-	Piraí do Sul/PR
Sisyrinchium sp.05	ESC497	-	Livramento/RS
Sisyrinchium sp.05	ESC515	-	Quaraí/RS
S. palmifolium	SP175	*Hydastylus*	Unknown origin
S. micranthum	SP839	*Sisyrinchium*	Colonia del

	Sacramento/Uruguay

MC = morphological category.

Table S2 – Primers used for amplifying and sequencing.

Region	Primer	Direction	Sequence of primer (5’ – 3’)
ITS	ITS-38f	F	CTGCGGAAGGATCATTGTC
	ITS4	R	TCCTCCGCTTATTGATATGC
trnQ-rps16	trnQ-rps16	F	GCGTGGCCAAGTGGTAAGGC
	Rps16-S1r	R	GTTGCTTTCTACCACATCGTTT
	trnQ-S801f	F	AACTCTTGATACTCGAGAAGAAGTG
	trnQ-S493r	R	TACGCCCGTTATTTGGACTTTC

Table S3 – Polymorphism showed by supported groups in ITS and *trnQ-rps16* markers. Regions marked with gray correspond to synapomorphic characters.

		ITS sites									
Accessions	Group	30	36	38-40	43	50-51	54	56-57	59-60	65	74
ESC571 *S. rambonis/* ESC351 *S. commutatum* ssp. *capilare*	I	A	G	_AA	T	TT	A	_C	CT	T	C
ESC245 *S. commutatum/* Group III	II	A	G	_AA	T,C	TT	A	_C	CT	C	C
ESC331, ESC366, ESC243 *S. commutatum*	III	A	G	_AA	C	TT	A	_C	CT,TT	C	C
ESC498, SP089, ESC277 *S. scariosum*	IV	A,C	G,A	_AA, _AG	C	TC	A	_C	CT	C	C
ESC214, ESC225 *S. setaceum*	V	C	G	_CG	C	TC	A	_C	CT	C	C
ESC400, ESC355, ESC391 *S. hoehneii*	VI	C	G	_CG	C	TC	A	_C	CT	C	C
ESC618, ESC373 *S. hoehneii*	VII	C	G	_CG	C	TA	A	CC	CT	C	C
ESC384 *S. luzula* ESC387 *S. purpurelum* ssp. *Purpurelum*	VIII	C	G	_CG	C	TC	A	_C	CT,GT	C	C

ESC349, ESC630 *Sisyrinchium* sp.04	IX	C	G	_CG	T	TC	A	_C	CT	C	C
ESC361 *S. cf. burchellii* ESC362 *Sisyrinchium* sp.02	X	C	G	_CG	T	TC	A	_T	CT	C	A
ESC337, LBC20, JMF4 *S. sellowianum* MCII	XI	C	G	_CG	C	TC	A	_C	CA	C	C
ESC363 *Sisyrinchium* sp.03 ESC246 *S.* aff. *luzula*	XII	C	G	_CG	T	TC	A	_C	CT	C	C
S. palmifolium	-	A	G	CCG	C	TT	C	_C	CT	T	C
S. micranthum	-	deletion	G	_CG	C	CT	A	_C	CT	T	C

Cont. Table S3 – Polymorphism showed by supported groups in ITS and *trnQ-rps16* markers. Regions marked with gray correspond to synapomorphic characters.

		ITS sites									
Accessions	Group	82	86	92	101-102	104	107	118	121-122	125	135
ESC571 *S. rambonis/* ESC351 *S. commutatum* ssp. *capilare*	I	G	C	T	AC	G	C	C	CC	G	_
ESC245 *S. commutatum*/ Group III	II	G	deletion	T	AC,AT	G	C	C	CC,CT	G	_
ESC331, ESC366, ESC243 *S. commutatum*	III	G	deletion	T	AC,AT	G	C	C	CC	G	_
ESC498, SP089, ESC277 *S. scariosum*	IV	G	deletion	T	AC	G	C,T	C	CC	G	_
ESC214, ESC225 *S. setaceum*	V	G	deletion	C	AC	G	C	C,T	CC	G	_
ESC400, ESC355, ESC391 *S. hoehneii*	VI	G	deletion	C	AC	G,A	C	C	CC	G	_
ESC618, ESC373 *S. hoehneii*	VII	G	deletion	C	AC	G	C	C	CC	G	_
ESC384 *S. luzula* ESC387 *S. purpurelum* ssp.	VIII	G	deletion	C	AC	G	C	C	TC	G	_

Purpurelum											
ESC349, ESC630	IX	G	deletion	T	AC	G	C	C	CC	G	_
Sisyrinchium sp.04											
ESC361 *S. cf. burchellii*	X	G	deletion	T	AC	G	C	C	CC	G	_
ESC362 *Sisyrinchium* sp.02											
ESC337, LBC20, JMF4	XI	G	deletion	T	GC	G	C	C	CC	A	_
S. sellowianum MCII											
ESC363 *Sisyrinchium* sp.03	XII	G	deletion	T	AC	G	C	C	CC	G	_
ESC246 *S.* aff. *luzula*											
S. palmifolium	-	G	C	T	AC	G	C	C	CC	G	_
S. micranthum	-	T	C	T	AC	G	C	C	CC	A	insertion C

Cont. Table S3 – Polymorphism showed by supported groups in ITS and *trnQ-rps16* markers. Regions marked with gray correspond to synapomorphic characters.

		ITS sites					
Accessions	Group	144	152	178-180	182-185	187-190	198-200
ESC571 *S. rambonis*/ ESC351 *S. commutatum* ssp. *capilare*	I	A	C	AAC	TCTG	_GTC	TTC
ESC245 *S. commutatum*/ Group III	II	A	C	AAC,ACC	TCCG,ACCA, ACCG	_GTC	TCC,ATC,TTC
ESC331, ESC366, ESC243 *S. commutatum*	III	A	C	AAC	ACCA,ACCG	_GTC	ATC,TTC
ESC498, SP089, ESC277 *S. scariosum*	IV	A	C	AAC,GAC	TTCG,TCCG	_GTA,_GTC	TTC
ESC214, ESC225 *S. setaceum*	V	A	T	AAC	TCCG,TCTG	_GTC	TTC
ESC400, ESC355, ESC391 *S. hoehneii*	VI	A	C	AAC	TCCG	_GTC	TTC
ESC618, ESC373 *S. hoehneii*	VII	A	C	AAC	TCCG	_GTC	TTC
ESC384 *S. luzula* ESC387 *S. purpurelum* ssp. *purpurelum*	VIII	A	C	AAC	TCCG	AATC	TTC
ESC349, ESC630	IX	A	C	AAT	TCCG	_GCC	TTC

Sisyrinchium sp.04							
ESC361 *S. cf. burchellii*	X	A	C	AAT	TCCG	_GCC	TTC
ESC362 *Sisyrinchium* sp.02							
ESC337, LBC20, JMF4	XI	A	C	AAC	ACCG	_GCC	TTC
S. sellowianum MCII							
ESC363 *Sisyrinchium* sp.03	XII	A	C	AAC	TCCG	_GCC	TTC
ESC246 *S.* aff. *luzula*							
S. palmifolium	-	G	C	AAC	TCCG	_GTC	TTA
S. micranthum	-	G	C	AAC	TCCG	_GTC	TTC

Cont. Table S3 – Polymorphism showed by supported groups in ITS and *trnQ-rps16* markers. Regions marked with gray correspond to synapomorphic characters.

		ITS sites						
Accessions	Group	208	212-215	217-219	221-222	229	234-235	240
ESC571 *S. rambonis*/ ESC351 *S. commutatum* ssp. *capilare*	I	A	ACGC	GGC	TG	G	_G	C
ESC245 *S. commutatum*/ Group III	II	A,G	ACGC	GGG	TG	G	_G	C
ESC331, ESC366, ESC243 *S. commutatum*	III	G	ACGC	GGG	TG	G	_G	C
ESC498, SP089, ESC277 *S. scariosum*	IV	A	ACGT,ACGC	GGG,G_G	TG,TT	G	_G	C
ESC214, ESC225 *S. setaceum*	V	A	ACGC	AGG,GGG	TG	G	_G	C
ESC400, ESC355, ESC391 *S. hoehneii*	VI	A	TCAC,GCAC	GGG	TG	G	_G	C
ESC618, ESC373 *S. hoehneii*	VII	A	GCGC	GGG	TG	G	_G	C
ESC384 *S. luzula* ESC387 *S. purpurelum* ssp. *purpurelum*	VIII	A	ATGC	GGG	TG	G	_G	C
ESC349, ESC630	IX	A	ACGT	GGG	TT	G	_G	C

Sisyrinchium sp.04								
ESC361 *S. cf. burchellii*	X	A	ACGT	GGG	TT	G	_G	C
ESC362 *Sisyrinchium* sp.02								
ESC337, LBC20, JMF4	XI	A	ACGC	GGG	TT	A	_G	C
S. sellowianum MCII								
ESC363 *Sisyrinchium* sp.03	XII	A	ACGT	GGG	CT	G	_G	C
ESC246 *S.* aff. *luzula*								
S. palmifolium	-	A	ACAC	GGG	TG	G	_A	G
S. micranthum	-	A	ACGC	GGG	TG	A	CG	G

Cont. Table S3 – Polymorphism showed by supported groups in ITS and *trnQ-rps16* markers. Regions marked with gray correspond to synapomorphic characters.

		ITS sites									
Accessions	Group	243	380	441	443	445	448-451	455	457	464	470
ESC571 *S. rambonis/* ESC351 *S. commutatum* ssp. *capilare*	I	G	A	T	T	C	GGAA	A	C	C	G
ESC245 *S. commutatum/* Group III	II	G	A	T	T,C	C	GGAA	A	C	C	T
ESC331, ESC366, ESC243 *S. commutatum*	III	G	A	T	C	C	GGAA	A	C	C	T
ESC498, SP089, ESC277 *S. scariosum*	IV	G	A	T	T	C	GGAA,GAGA	A,C	A,C	C	G,T
ESC214, ESC225 *S. setaceum*	V	G	A	T	T	C	GGAA	C	C	C	G
ESC400, ESC355, ESC391 *S. hoehneii*	VI	G	A	T	T	C	GGAG	C	T	C	G
ESC618, ESC373 *S. hoehneii*	VII	G	A	T	T	C	GGAG	C	T	C	G
ESC384 *S. luzula* ESC387 *S. purpurelum* ssp. *purpurelum*	VIII	G	A	T	T	C	GGAA	C	A	C	G

ESC349, ESC630 *Sisyrinchium* sp.04	IX	G	A	T	T	C	GGAA	C	C	C	G
ESC361 *S. cf. burchellii* ESC362 *Sisyrinchium* sp.02	X	G	A	T	T	C	GGAA	C	C	T	G
ESC337, LBC20, JMF4 *S. sellowianum* MCII	XI	G	A	T	T	C	GGAA	T	C	C	G
ESC363 *Sisyrinchium* sp.03 ESC246 *S.* aff. *luzula*	XII	G	A	T	T	C	GGAA	C	C	C	G
S. palmifolium	-	G	G	T	T	T	AGAA	A	C	C	G
S. micranthum	-	T	A	C	T	T	GGAT	A	C	C	G

Cont. Table S3 – Polymorphism showed by supported groups in ITS and *trnQ-rps16* markers. Regions marked with gray correspond to synapomorphic characters.

		ITS sites									
Accessions	Group	479	484	487-488	503	507	521	523	533	577	583-585
ESC571 *S. rambonis/* ESC351 *S. commutatum* ssp. *capilare*	I	T	T	TC	T	T	G	A	A	G	GCA
ESC245 *S. commutatum/* Group III	II	T	G	TA	C	T	G	A	A	G, deletion	GCT
ESC331, ESC366, ESC243 *S. commutatum*	III	T	G	TA	T,C	T	G	A	A	deletion	GCT
ESC498, SP089, ESC277 *S. scariosum*	IV	T	G	TA	T	T,C	G	A	A	G	TCA,GCT
ESC214, ESC225 *S. setaceum*	V	T	G	CA	T	T	G	A	A	G	TCA
ESC400, ESC355, ESC391 *S. hoehneii*	VI	T	G	TA	T	T	G	A	A	G	TCA
ESC618, ESC373 *S. hoehneii*	VII	T	G	TA	T	T	G	A	A	G	TCA
ESC384 *S. luzula* ESC387 *S. purpurelum* ssp. *purpurelum*	VIII	T	A	TA	T	T	G,A	A	A	G	TCA

ESC349, ESC630 *Sisyrinchium* sp.04	IX	T	G	TA	T	T	G	A	A	G	TCA
ESC361 *S. cf. burchellii* ESC362 *Sisyrinchium* sp.02	X	T	G	TA	T	T	G	A	A	G	TCA
ESC337, LBC20, JMF4 *S. sellowianum* MCII	XI	T	G	TA	T	T	G	A	A	G	TCA
ESC363 *Sisyrinchium* sp.03 ESC246 *S.* aff. *luzula*	XII	T	G	TA	T	T	G	A	A	G	TCA
S. palmifolium	-	C	G	TC	T	T	G	G	G	G	GCA
S. micranthum	-	T	T	TA	T	T	G	G	G	G	GAT

Cont. Table S3– Polymorphism showed by supported groups in ITS and *trnQ-rps16* markers. Regions marked with gray correspond to synapomorphic characters.

		ITS sites									
Accessions	Group	591	594-595	602	606	617-618	623-624	637	656	680	691
ESC571 *S. rambonis*/ ESC351 *S. commutatum* ssp. *capilare*	I	A	AC	G	C	AT	GG	G	A	A	T
ESC245 *S. commutatum*/ Group III	II	A	AC	G	C	AT	GT,GG	A	A	A,G	T
ESC331, ESC366, ESC243 *S. commutatum*	III	A	AC	G	C	AT	GG	A	A	A,G	T
ESC498, SP089, ESC277 *S. scariosum*	IV	A,G	GT,AC	G,A	C	AT	AG	G,A	A	A	T
ESC214, ESC225 *S. setaceum*	V	A	GT	G	C	AT	GG	G	A	A	T
ESC400, ESC355, ESC391 *S. hoehneii*	VI	A	GT	G	C	AT	GG	G	A	A	T
ESC618, ESC373 *S. hoehneii*	VII	A	GT	G	C	AT	GG	G	A	A	T
ESC384 *S. luzula* ESC387 *S. purpurelum* ssp. *purpurelum*	VIII	A	TC	G	C	AT	GG	G	A	A,G	T

ESC349, ESC630 *Sisyrinchium* sp.04	IX	A	GT	G	C	AT	GG	G	A	A	T
ESC361 *S. cf. burchellii* ESC362 *Sisyrinchium* sp.02	X	A	GT	G	C	AT	TG	G	A	A	T
ESC337, LBC20, JMF4 *S. sellowianum* MCII	XI	A	GT	G	C	AT	GG	G	A	G	A
ESC363 *Sisyrinchium* sp.03 ESC246 *S.* aff. *luzula*	XII	A	GT	G	C	AT	GG	G	A	A	T
S. palmifolium	-	A	AC	G	T	TT	GG	A	A	N	N
S. micranthum	-	A	TC	G	C	TA	GG	T	G	N	N

Cont. Table S3 – Polymorphism showed by supported groups in ITS and *trnQ-rps16* markers. Regions marked with gray correspond to synapomorphic characters.

		trnQ-rps16 sites								
Accessions	Group	125	131	144	151	171-172	222-223	236-249	263	319
ESC571 *S. rambonis/* ESC351 *S. commutatum* ssp. *capilare*	I	deletion, A	G	C	G	TA	CC	deletion	T	A,C
ESC245 *S. commutatum/* Group III	II	deletion	G	C	G	TA	CC	deletion	T	A
ESC331, ESC366, ESC243 *S. commutatum*	III	deletion	G	C	G	TA	CC	deletion	T	A
ESC498, SP089, ESC277 *S. scariosum*	IV	A	G	C	G	TA	CC,CT	deletion	T	A
ESC214, ESC225 *S. setaceum*	V	A	G	C	G	TA	CC	deletion	T	A
ESC400, ESC355, ESC391 *S. hoehneii*	VI	A	G	C	G	TA	CC	deletion	T	A
ESC618, ESC373 *S. hoehneii*	VII	A	G	C	G	TA	CC	deletion	T	A
ESC384 *S. luzula* ESC387 *S. purpurelum* ssp. *purpurelum*	VIII	A	G	C	G	TA	CC	deletion	T	A
ESC349, ESC630	IX	A	G	C	G	TA	CC	deletion	T	A

Sisyrinchium sp.04											
ESC361 *S. cf. burchellii*	X	A	G	C	G	TA	CC	deletion	T	A
ESC362 *Sisyrinchium* sp.02											
ESC337, LBC20, JMF4	XI	A	G	C	G	TA	CC	deletion	T	A
S. sellowianum MCII											
ESC363 *Sisyrinchium* sp.03	XII	A	G	C	G	TA	CC	deletion	T	A
ESC246 *S.* aff. *luzula*											
S. palmifolium	-	A	T	A	A	deletion	CA	ATCAAATCA	C	A
S. micranthum	-	A	G	C	G	TA	TC	TTTTAATCAAATCC	T	A

Cont. Table S3 – Polymorphism showed by supported groups in ITS and *trnQ-rps16* markers. Regions marked with gray correspond to synapomorphic characters.

		trnQ-rps16 sites									
Accessions	Group	325	428-434	452	521	531	535	584	614	652-656	680
ESC571 *S. rambonis*/ ESC351 *S. commutatum* ssp. *capilare*	I	_	TC____G,CC____G	C	G,T	A	A	T	A	_____	_
ESC245 *S. commutatum*/ Group III	II	_	TC____G	C	G	A,C	A,C	T	A	_____	_
ESC331, ESC366, ESC243 *S. commutatum*	III	_	TC____G	C	G	A,C	A,C	T	A	_____	_
ESC498, SP089, ESC277 *S. scariosum*	IV	_	TC____G,CC____G	C,T	T	A	A	T	A	_____	_
ESC214, ESC225 *S. setaceum*	V	insertion A	CC____G	C	T	A	A	T	A	_____	_
ESC400, ESC355, ESC391 *S. hoehneii*	VI	_	CC____G	C	T	A	A	T	A	_____	_
ESC618, ESC373 *S. hoehneii*	VII	_	CC____G	C	T	A	A	T	A	_____	_
ESC384 *S. luzula* ESC387 *S. purpurelum* ssp. *purpurelum*	VIII	_	CC____G	C	T	A	A	T	A	_____	_

ESC349, ESC630 *Sisyrinchium* sp.04	IX	_	CC____G	C	T	A	A	T	A	_____	_
ESC361 *S. cf. burchellii* ESC362 *Sisyrinchium* sp.02	X	_	CC____G	C	T	A	A	T	A	_____	_
ESC337, LBC20, JMF4 *S. sellowianum* MCII	XI	_	CC____G	C	T	A	A	T	A	_____	insertion A
ESC363 *Sisyrinchium* sp.03 ESC246 *S.* aff. *luzula*	XII	_	CC____G	C	T	A	A	T	A	_____	_
S. palmifolium	-	_	CCCATTA	C	T	A	A	C	A	_____	_
S. micranthum	-	_	C_____G	C	T	A	A	T	G	TAATT	_

Cont. Table S3 – Polymorphism showed by supported groups in ITS and *trnQ-rps16* markers. Regions marked with gray correspond to synapomorphic characters.

		trnQ-rps16 sites							
Accessions	Group	697-698	706-713	776	785	788-792	828-832	871-878	905-906
ESC571 *S. rambonis/* ESC351 *S. commutatum* ssp. *capilare*	I	__	deletion	A,G	C	ATAAT	A___C	deletion	TA
ESC245 *S. commutatum*/ Group III	II	__	deletion	G	C	ATAAT	A___C	deletion	TA
ESC331, ESC366, ESC243 *S. commutatum*	III	__	deletion	G	C	ATAAT	A___C	deletion	TA
ESC498, SP089, ESC277 *S. scariosum*	IV	__	deletion	A	C,A	ATAAT	A___C	deletion	TA
ESC214, ESC225 *S. setaceum*	V	__	deletion	A	C	ATAAT	A___C,A___A	deletion	TA
ESC400, ESC355, ESC391 *S. hoehneii*	VI	Insertion AT __	deletion	A	C	ATAAT	A___C	deletion	TA
ESC618, ESC373 *S. hoehneii*	VII	__	deletion	A	C	ATAAT	A___C	deletion	TA
ESC384 *S. luzula* ESC387 *S. purpurelum* ssp. *purpurelum*	VIII	__	deletion	A	C	ATAAT	A___C	deletion	TA
ESC349, ESC630	IX	__	deletion	A	C	ATAAT	A___C	deletion	TA

Sisyrinchium sp.04									
ESC361 *S. cf. burchellii*	X	__	deletion	A	C	ATAAT	A___C	deletion	TA
ESC362 *Sisyrinchium* sp.02									
ESC337, LBC20, JMF4	XI	__	deletion	A	C	ATAAT	A___C	deletion	TA
S. sellowianum MCII									
ESC363 *Sisyrinchium* sp.03	XII	__	deletion	A	C	ATAAT	A___C	deletion	TA
ESC246 *S.* aff. *luzula*									
S. palmifolium	-	__	TTCTTTTG	A	C	ATAAT	ATATC	AAATGAAC	AT
S. micranthum	-	__	deletion	A	A	DEL	DEL	AAATGAAC	TA

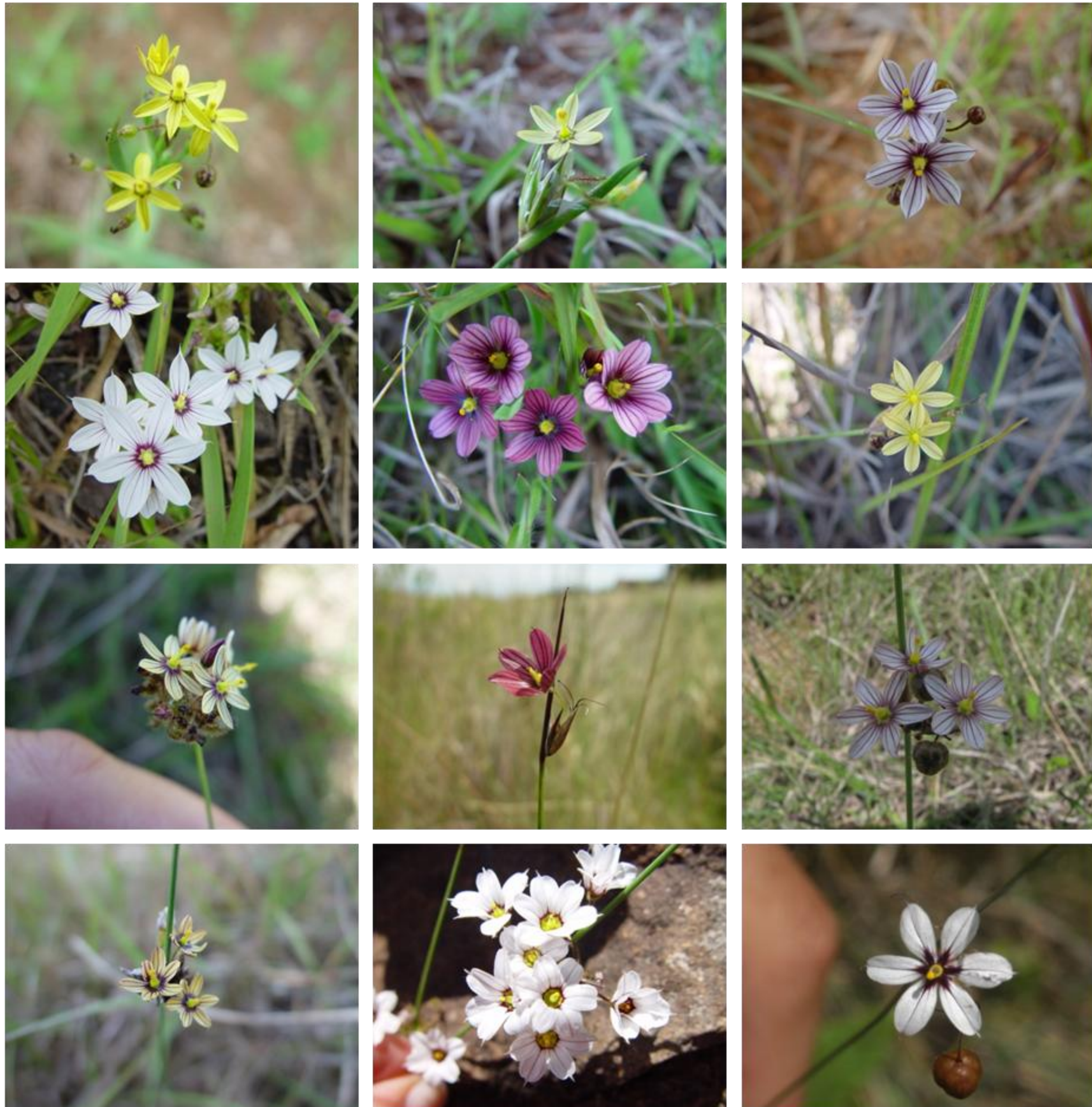
Cont. Table S3 – Polymorphism showed by supported groups in ITS and *trnQ-rps16* markers. Regions marked with gray correspond to synapomorphic characters.

		trnQ-rps16 sites			
Accessions	Group	909	1057	1064	1067
ESC571 *S. rambonis/* ESC351 *S. commutatum* ssp. *capilare*	I	G	T	A	A
ESC245 *S. commutatum/* Group III	II	G	T	A	A
ESC331, ESC366, ESC243 *S. commutatum*	III	G	T	A	A
ESC498, SP089, ESC277 *S. scariosum*	IV	G	T	A	A
ESC214, ESC225 *S. setaceum*	V	G	T	A	A
ESC400, ESC355, ESC391 *S. hoehneii*	VI	G	T	A	A
ESC618, ESC373 *S. hoehneii*	VII	G	T	A	A
ESC384 *S. luzula* ESC387 *S. purpurelum* ssp. *purpurelum*	VIII	G	T, deletion	A	A
ESC349, ESC630	IX	G	T	A	A

Sisyrinchium sp.04					
ESC361 *S. cf. burchellii*	X	G	T	A	A
ESC362 *Sisyrinchium* sp.02					
ESC337, LBC20, JMF4	XI	G	T	A	A
S. sellowianum MCII					
ESC363 *Sisyrinchium* sp.03	XII	G	T	A	A
ESC246 *S.* aff. *luzula*					
S. palmifolium	-	C	T	C	A
S. micranthum	-	G	T	C	C

CAPÍTULO III

Genetic variation, population structure, gene flow and chromosome number analysis in the clade V of the genus *Sisyrinchium* L.



As espécies pertencentes ao clado V de *Sisyrinchium* possuem folhas do tipo terete ou cilíndricas, e uma grande diversidade de formas e cores das flores. Fotos: Lilian Eggers

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GENETIC VARIATION, POPULATION STRUCTURE, GENE FLOW AND CHROMOSOME NUMBER ANALYSIS OF THE CLADE V OF THE GENUS *SISYRINCHIUM* L. (IRIDACEAE)[1]

Juliana Fachinetto[2], Eliane Kaltchuk-Santos[2], Lilian Eggers[3],
Tatiana Teixeira de Souza-Chies[2,3,4].

[2]Departamento de Genética and [3]Departamento de Botânica, Universidade Federal do Rio Grande do Sul, CEP 91501-970, Porto Alegre, Rio Grande do Sul, Brazil

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The authors thank CNPq and Capes for financial support.

[4]E-mail: tatiana.chies@ufrgs.br

Premise of the study: Species of the genus *Sisyrinchium* occur in American continent and present considerable morphological variation in natural populations. Phylogenetic analysis with the species of the clade V of *Sisyrinchium* indicated low nucleotide diversity to separate the species. The aim of this study was assess the levels of genetic variation by dominant molecular markers to verify the inter-specific relation. In addition, analyze the population structure and gene flow among species and determine the chromosome number of the species of the clade V of *Sisyrinchium*.

Methods: Twenty eigth populations belonging to eleven species of *Sisyrinchium* were analyzed through ISSR markers. In addition, the chromosome number for these species was determined.

Key Results: An amount of 104 fragments was produced, of which 97.12% were polymorphic. The populations are strongly structured ($F_{ST}= \theta^B= 0.51$) and when species were analyzed separately, the values of F_{ST} ranged from 0.38 (*S. sellowianum*) to 0.73 (*S. hoeneii*). The Mantel test showed no significant correlation between genetic and geographic distance (r= 0.14, p= 0.100). However, the high population structure can be explained by the distance among populations, which possibly reduces gene flow due to the behavior of pollinators. Different intra-specific ploidy levels were observed in some species with diploid (2n= 2x= 18 chromosomes), tetraploid (2n= 4x= 36 chromosomes) and hexaploid (2n= 6x= 54 chromosomes) populations.

Conclusions: The populations showed high genetic variation within and strong structuring. The sympatric species presented low gene flow, indicating that there is some barrier to the crossing. Polyploidy seems has contributed to the evolution of these species, as well as to other species of *Sisyrinchium*.

Keywords: interspecific diversity; Iridaceae; ISSR; polyploidy.

Species groups with complicated phylogenetic relationships, evolutionary history and intraspecific variation are not rare among vascular plants. Due to the ambiguity of the intraspecific variation patterns observed, taxa circumscription and delimitation are often surrounded by controversy, and taxonomic concepts are difficult to establish (Mereda et al., 2008). Moreover, phylogenetic reconstruction for such groups employing DNA sequence data often represents a major challenge because there usually is not sufficient variation in the sequences of commonly employed regions (Archibald et al., 2006). While there is a continual search for more variable gene regions to sequence (Mort and Crawford, 2004; Small et al., 2004; Shaw et al., 2005), there has also been increasing interest in the use of hypervariable, arbitrarily amplified dominant markers (e.g., AFLP, ISSR, RAPD). Inter-simple sequence repeats (ISSR) markers have been used for relationships analyses involving species that are closely related and represent recent radiations at the population level (Agostini et al., 2008; Archibald et al., 2006; Li et al., 2008), as well to investigate the intra-population diversity (Borón et al., 2011; Burgos et al., 2011; Tacuatiá et al., 2012; Zhang et al., 2006), the clonal diversity (Li et al., 2006) and even conservation purpose (George et al., 2009; Luan et al., 2006; Wei et al., 2008). In Iridaceae, ISSR markers have been used to analyze the genetic variability and population structure for several species of southern Brazil. In *Sisyrinchium micranthum* Cav. the molecular analysis showed that the populations are highly structured with low gene flow among them (F_{ST}= 0.33) (Tacuatiá et al., 2012). The same occur in others species of *Sisyrinchium, S. palmifolium, S. sellowianum* and *S. vaginatum* (F_{ST}= 0.47, F_{ST}= 0.43 and F_{ST}= 0.45, respectively) (Souza-Chies et al., 2012). In *Calydorea crocoides* Ravenna, although the species presents a very limited geographical distribution also was found high population structure with low gene flow (F_{ST}= 0.24) (Souza-Chies et al., 2012). Only in *Cypella fucata* Ravenna, the data indicated gene flow between the populations (F_{ST}= 0.0851) and high identity between the two collections sites (98%) (De Marco et al., 2009).

Sisyrinchium L. is the second largest genus of the family Iridaceae, represented by approximately 140 species in America (Goldblatt et al., 2008; Goldblatt and Manning, 2008). South America is the center of origin and distribution of the genus (Chauveau et al., 2011). This genus is considered of complicated taxonomy (Cholewa and Henderson, 1984; Kenton et al., 1986; Rudall et al., 1986) mainly due to the wide morphological variation of individuals in natural populations, frequently associated to polyploidization events and

breeding systems, an example, is *S. micranthum* Cav. (Tacuatiá et al., 2012).

Trichomal elaiophores are particularly widespread in about 35% of the species of *Sisyrinchium* (Chauveau et al., 2011). They produce non-volatile oils gathered by bees belonging to the families Apideae and Mellitidae, which have developed morphological adaptations on their legs or abdomen to harvest and store lipids (Cocucci and Vogel, 2001). Trichomatic elaiophores are located either on the staminal column or on the adaxial side of the tepals, sometimes on both parts, a distribution pattern that has only been observed on flowers of this genus (Goldblatt and Manning, 2008; Chauveau et al., 2011).

Chauveau et al. (2011) in a recent phylogenetic analysis of S*isyrinchium* suggested that floral glandular trichomes evolved three times independently in the genus and the species are clustered in nine clades. The clade V, object of this study, grouped species with trichomes on the filamental column and oil secretion. The species of this clade belong to two morphological sections, *Lenitium* and *Scirpeocaris* (Ravenna, 2000, 2002a, 2002b, 2003a, 2003b). A phylogenetic analysis using only species of this clade indicated low nucleotide diversity, which was inconclusive for understanding the inter-specific phylogenetic relationships (Fachinetto et al., unpublished).

In this study, ISSR markers were used in order to assess levels of genetic variation to verify the inter-specific relation and to determine the degree of differentiation of natural populations and species belonging to the clade V of *Sisyrinchium* (Chauveau et al., 2011). In addition, the groups of these different species were investigated and the gene flow between species in sympatric areas, using the ISSR markers. The chromosome numbers and ploidy levels were also determined for the species focused in this study.

MATERIAL AND METHODS

Study species **–** Eleven species of *Sisyrinchium* belonging to the clade V (Chauveau et al., 2011) were sampled in southern Brazil, totalizing 28 populations. Distribution of species, species, collections sites and vouchers are shown in Figure 1 and Table 1. Voucher specimens were deposited in the ICN Herbarium, Instituto de Biociências, Universidade Federal do Rio Grande do Sul, Porto Alegre, Rio Grande do Sul, Brazil. The species referred as *Sisyrinchium* sp (white) is probably a new species, which morphological characterization in ongoing and will be published later.

Plant materials, DNA extraction and ISSR-PCR amplification **–** Silica-dried samples of

leaf material were obtained from 24 populations of eight species of *Sisyrinchium*. A total of 633 individuals (nine to 33 individuals per population; Table 1) was analyzed.

Total genomic DNA was extracted based on the Doyle and Doyle method (1987) with modifications. DNA concentrations were determined by spectrophotometry (Nanodrop®). A set of fourteen ISSR primers (Agostini et al., 2008) was tested, and four of them generated good patterns for the eight species and were used for DNA amplification of all populations (Table 2).

PCR was carried out in 25 uL reactions using (depending on the primer): 0-4% DMSO, 1x buffer Taq DNA polymerase (Cenbiot, Rio Grande do Sul, Brazil), 4.0-5.0 mM $MgCl_2$, 0.8 mM dNTP (Invitrogen, São Paulo, Brazil), 0.8 mM of primer, 1U *Taq* DNA polymerase (CenBiot, Rio Grande do Sul, Brazil), 20-30 ng of genomic DNA and completed with water in a termal cycler Veriti 96 Well Thermal Cycle, Applied Biosystems. The thermal cycling program for amplification consisted of initial denaturing at 94 °C for 5 min, followed by 40 cycles of denaturation at 94 °C for 1 min, annealing at 48°C for 1 min, extension at 72°C for 2 min 30s, and a final extension step at 72 °C for 5 min. PCR products were analyzed on 1.5% agarose gels and stained with GelRed (Amicon Corp., Lexington, MA).

Statistical analyses – Amplified ISSR bands were scored for each individual as binary presence (1) or absence (0) characters. The resulting data matrix was analyzed using POPGENE v. 1.32 (Yeh et al., 2000) to estimate four genetic diversity parameters: percentage of polymorphic loci (P), Shannon's Informations Index (*I*), Nei's gene diversity (*H*) (Nei, 1973) and gene flow estimates (Nm) calculated as $Nm = 0.5\ (1\text{-}G_{st})/G_{st}$ (McDermott and McDonald, 1993). These parameters were analyzed for all 24 populations, for each species and for each population.

To infer population genetic structure and differentiation among populations, we ran an analysis of molecular variance (AMOVA; Excoffier et al., 1992) using the program ARLEQUIM version 3.5 (Excoffier and Lischer, 2010). After, populations were grouping according to the species to which they belong. To evaluate if there is differentiation among populations or groups of populations and among populations within groups, the *F* statistics was performed (i.e., F_{ST}, F_{CT} and F_{SC}). It was also calculated the F_{ST} values for each species separately.

An unbiased genetic distance matrix (Nei, 1978) was generated by TFPGA version 1.3

(Tools for Population Genetic Analyses; Miller, 1997) to construct an unweighted pair-group method arithmetic average (UPGMA) topology, which computed 1000 permutations and estimated the confidence limits of the dendrogram. Marker frequencies were estimated based on the Lynch and Milligan (1994) Taylor expansion estimate.

To assess the genetic similarity of individuals across populations, Jaccard similarity coefficients for all pairs of individuals and an UPGMA dendrogram was generated using the program NTSYSpc version 2.1N (Rohlf, 2001).

A Principal Components Analysis (PCA) was performed in Past version 2.15 (Hammer et al., 2001) to recover the non-hierarchical structure in the dataset. The dataset was formed by populations of *S. luzula* and the populations of the species morphologically more similar, as *S. scariosum*, *S.* cf. *claritae* and *Sisyrinchium* sp. (white).

To test the correlation between the unbiased genetic distance matrix generated by TFPGA and geographic distances (in km) among populations, a Mantel Test was performed using GenAlEx population genetics package (Peakall and Smouse, 2006) with 9999 permutations.

A Bayesian approach proposed by Holsinger et al. (2002) was also applied with HICKORY version 1.1, to obtain a more direct estimate of F_{ST} from dominant markers, unaffected by Hardy-Weinberg and f assumptions. The *a posteriori* distribution of the θ^B estimator (the estimate F_{ST}) was numerically approximated through a Markov Chain Monte Carlo (MCMC) simulation, and tends to converge to a beta distribution (see Telles et al., 2006). The four models available in the software were tested, i.e., the full model which allows estimating both θ^B and f, models with f or θ^B equal to zero, and, a final model leaving f free to vary so that the sampler does not attempt to estimate f, but chooses f values from its prior distribution, while estimating other parameters during the MCMC run. Model choice was based on the Deviance Information Criterion (DIC; Spiegelhalter et al., 2002). Estimates of genetic diversity (hs; defined as average panmictic heterozygosity) within each population were also calculated.

STRUCTURE version 2.3.1 (Falush et al., 2007) was employed to obtain additional insights regarding gene flow and population subdivision in the sympatric areas. Five sites where populations of different species occur in sympatry were evaluated, corresponding to ESC 245/246 (*S. commutatum* Klatt./*S. luzula* Klotzsch ex Klatt.), ESC331/625 (*S. commutatum/S. luzula*), ESC 387/388 (*S. luzula/S. sellowianum* Klatt.), ESC497/499

(*Sisyrinchium* sp (white)/*S. claritae* Herter.) and ESC 689/690 (*S. luzula/S. setaceum* Klatt.). The most likely number of populations (*k*) was estimated under the admixture model and correlated allele frequencies, with no prior information on population origin. The program was run for 10,000 iterations, after a burn-in length of 10,000 iterations, to test population subdivision from k=1 to k=4 to each site, and thereby check for any possible subdivision. Twenty runs were carried out for each *k*, to quantify variation in likelihood, as a means of checking whether different runs could produce different likelihood values. Individual and average admixture proportions (*Q*) for each population in each genetic cluster found by the program, were recorded for the model. As an aid in identifying the number of clusters of individuals (*k*), the results generated by STRUCTURE were subsequently analyzed by STRUCTURE HARVESTER version 0.6.7 (Earl, 2011), according to the method of Evanno et al. (2005). This method uses an *ad hoc* statistic *k*, based on the rate of change in the log probability of data between successive *k* values (see Evanno et al., 2005, for a better explanation).

Determination of chromosome number – Young inflorescences of eighteen accessions belonging to ten species from *Sisyrinchium* (Table 1) were collected and fixed in ethanol: acetic acid (3:1) for 24 h at room temperature and stored at -4°C. The slides for analysis were prepared by anthers squashing and staining with propionic carmin 1%. Approximately 10 pollen mother cells in diakinesis or anaphase I were analyzed to determine the chromosome number of each accession.

RESULTS

A total of 104 ISSR computable fragments were produced, of which 97.12% were polymorphic. The ISSR markers generated an average of 26 fragments per primer and their size ranged from 200 to 2080 pb (Table 2).

The population ESC 245 (*S. commutatum*) showed the lowest values of polymorphic loci (14.42), Shannon's Information Index (0.07) and Nei's gene diversity (0.05) whereas ESC 625 (*S. luzula*) the highest (55.77, 0.25 and 0.16), respectively. At the species level, *S. commutatum* showed the lowest values (I = 0.15, H = 0.10), and *S. luzula* the highest (I = 0.30, H = 0.19) (Table 3). The gene flow estimates suggest that exchange among species is limited (Nm = 0.54), only the species *S. scariosum* Johnst., *S. sellowianum* and *S. setaceum* had more than one migrant per generation (Table 4).

Population structure determined by AMOVA showed 51.34% of the total variation was due to variation among populations, and 48.66% was attributed to differences among individuals within populations (Table 5), resulting in F_{ST}= 0.51 (Table 4). When populations were grouped by species, the proportion of total variance residing among populations was identical (i.e. F_{ST}= 0.51), with only 7.48% of total variance among groups (Table 5).

When species were analyzed separately, the values of F_{ST} ranged from 0.38 (*S. sellowianum*) to 0.73 (*S. hoeneii* Johnst.) (Table 4). In regarding the distribution of the total variation, the species *S. commutatum, S. hoeneii, S. setaceum* and *Siyrinchium* sp. (white) showed more divergence among populations, contrasting with *S. luzula, S. scariosum* and *S. sellowianum* in which the variation was higher within populations (Table 5).

The UPGMA dendrogram produced by TFPGA (Figure 2) based on the Nei's unbiased genetic distance matrix presented two main clusters, one comprising only the ESC 355 (*S. hoeneii*) and the second the remaining populations. The other branches were not supported by Bootstrap. In regarding to the grouping intra-specific, only the four populations of *S. sellowianum* clustered. The other accessions were not grouped according to species.

These results were highly concordant with the PCA results (Figure 3). In PCA results, the populations of the *S. luzula* and species similar do not grouped in according to species.

MC-I were highly related between them, while in *S. sellowianum* MC-II, the populations are more distant from each other. The individuals of each population of *S. sellowianum* MC-II are highly related, reflecting strong population structure in this category. In addition, the populations of *S. sellowianum* MC-II formed two groups, ESC337/ LBC20 and ESC686/ JMF3/ JMF4, identical to the dendrogram UPGMA obtained from TFPGA. The two morphological categories are distanced from each other.

The UPGMA dendrogram generated by NTSYS revealed that individuals were grouped according to their populations, in agreement with the high values of F_{ST} (Figure 4).

The Mantel test showed no significant correlation between geographical and genetic distances (r = 0.14, p= 0.100).

According to population analysis performed with HICKORY, the best model, i.e., which present lowest DIC (DIC= 5368.73), was the full model, with θ^B= 0.51 and f= 0.97. θ^B value was identical to F_{ST} presented by AMOVA (Table 4). At the species level, θ^B ranged from 0.42 (*Sisyrinchium sp* (white)) to 0.64 (*S. hoeneii*), while *f* from 0.39 (*S. luzula*) to

0.77 (*S. sellowianum*). In relation to genetic diversity (hs), the values ranged from 0.06 (ESC 355, *S. commutatum*) to 0.19 (ESC 625, *S. luzula*), with an average of 0.13 (Table 3). At the species level, the lowest value of hs was 0.14 (*S. commutatum*), while the highest was 0.23 (*S. scariosum, S. setaceum* and *Sisyrinchium* sp. (white)).

The five sites where species of *Sisyrinchium* occur in sympatry, analyzed by STRUCTURE, revealed *k*= 2 model the most adequate. In all cases, low gene flow was evidenced by admixture (Figure 5).

Chromosome numbers of eighteen accessions were analyzed (Table 1 and Figure 6). The ten species presented the same basic number x=9, and three ploidy levels were observed: diploid, tetraploid and hexaploid. *S. luzula*, which had six populations analyzed, presented three possible cytotypes, varying according to ploidy levels (2x, 4x and 6x). The diploids and tetraploids are more frequent, with only two hexaploid accessions, among those analyzed in this study.

DISCUSSION

The ISSR markers detected from 24 populations belonging to 8 species of *Sisyrinchium* revealed a high level of genetic variation. Genetic diversity of the plant species reflects their breeding systems (Hamrick and Godt, 1996). In general, outcrossing species commonly display higher levels of genetic diversity and lower differentiation between populations than selfing and clonal plants (Rossetto et al., 1995). *S. commutatum* showed the lowest values of genetic variation (P= 37.50; *I*= 0.15; *H*= 0.10; hs= 0.14), suggesting selfing. Self-pollination is highly probable when the anther is at the same level of the style. Even so, outcrossing may occur when the filament column is shorter than the style (Ingram, 1968). However, floral morphology of this and all other species studied here suggests outcrossing. In addition, decreased gene diversity still can occur, if several flowers on an inflorescence are receptive at the same time and pollinators visit consecutive flowers on an inflorescence and return to plants previously visited (George et al., 2009). The species of *Sisyrinchium*, as in *S. commutatum*, can produce a large number of flowers per plant, which can influence the genetic diversity index. Decreased genetic diversity can also occur if the proportion of flowering plants is low. In *Piperia yadonii* Rand. Morgan & Ackerman found low expected heterozygosity (Nei’s gene diversity) within populations that is related to the proportion of flowering plant (George et al., 2009).

The analyses inferred from ISSR markers using different approaches (AMOVA and Bayesian analysis) demonstrated a high interpopulation differentiation (F_{ST}= θ^B= 0.51) to the species of *Sisyrinchium* (Table 4). In addition, all species analyzed separately, showed strong population structuring. The AMOVA indicated that 51.33% of the total genetic variation was partitioned among populations. When the populations were grouped according to the species to which they belong, only 7.48% of total variance resided among species (Table 5). These results demonstrate that the high degree of population differentiation is more related to differences among populations than among species or individuals. The populations analysed shown high population structure when all populations were analyzed together and when the populations were analyzed separately by species, both analysis revelead F_{ST}= θ^B= 0.51. With exception of *S. luzula*, *S. scariosum* and *S. sellowianum*, the other species exhibit larger genetic variation among population than within population. These results can reflect a reduction of gene flow in these species, which may be due to different factors such as habitat fragmentation, reduction of pollinators, founder effects, genetic drift or mating system.

Highly structured populations were also found in *Piperia yadonii*, likely due to habitat fragmentation and limited gene flow (George et al., 2009). On the other hand, in *Tacca chantrieri* Andre high levels of differentiation among populations and low levels of diversity within populations suggest the occurrence of a mixed-mating system in which self-fertilization predominates, but there is occasional outcrossing (Zhang et al., 2005; Zhang et al., 2006). In *Croomia japonica* Miq. and *Croomia heterosepala* (Bak.) Oku. it was detected remarkably low levels of variation within-population and high levels of inter-population differentiation, at least partly due to pronounced regional genetic substructure within both species (Li et al., 2008). Tacuatiá et al. (2012) verified in *S. micranthum* a high population structure probably due to the breeding system associated with polyploidy. In this study, a high population structure was found beyond the occurrence of polyploidy in species of *Sisyrinchium* analyzed, which, however, do not seem to be related to breeding system.

A high level of population differentiation may be explained by several factors, such as species breeding system, genetic drift, demographic fluctuations, or the genetic isolation of populations (Hogbin and Peakall, 1999). When populations are small and geographically and genetically isolated from each other, genetic drift influences the genetic structure and

increases differentiation among populations (Barrett and Kohn, 1991; Ellstrand and Elam, 1993). Estimates of the effective gene flow per generation (*Nm*) in *Sisyrinchium* species (Table 4) were lower than one migrant per generation (0.54), indicating limited gene flow among species. Even when the species were analyzed separately, only *S. scariosum* (1.63), *S. sellowianum* (1.19) and *S. setaceum* (1.04) had more than one migrant per generation, which may be insufficient to counteract the effect of genetic drift. The places of collection are, in most cases, separated by areas of agriculture production and human occupacion, which can reduce gene flow by fragmentation of habitats. Gene flow can be detected by the amount of pollen flow, but also includes movement of seeds and, more rarely in terrestrial plants, movement of individuals (Batista and Sosa, 2002). In addition, *Nm* may also represent historical genetic exchange and ancestral polymorphism retention, and are not indicative of current migration rates (Luan et al., 2006).

In addition, the low gene flow could be associated with pollinator behavior. The most probable pollinators of species of S*isyrinchium* with elaiophores, are oil-bees from the family Apidae, of the tribe Tapinotaspidini (Cocucci and Vogel, 2001; Truylio et al., 2002) which usually nest close to foraging areas. Furthermore, there is a great geographical distance among populations for each species, making difficult foraging pollinator. However, the genetic resemblance and the geographical proximity did not appear significantly related in this study, as shown by the non significance of mantel test (r= 0.14, p= 0.100). The closer geographically populations were not genetically closer.

The UPGMA dendrogram produced by TFPGA (Figure 2) based on the Nei's unbiased genetic distance matrix and PCA (Figure 3) does not discriminated the different species. However, the UPGMA dendrogram generated by NTSYS grouped the individuals according to their populations (Figure 4). These results are in agreement with those obtained by Archibald et al. (2006); these authors showed that ISSR markers are most useful in *Tolpis* Adanson. for grouping individuals into populations and species. According to these authors, ISSR markers fail to group populations in species that are self-incompatible, morphologically variable, and with large population sizes. The lack of clustering of these members of *Sisyrinchium* may also be the result of the presence of a suite of ancestral polymorphic markers segregating within populations referable to more than one species in the current taxonomy of the genus (Jarvis, 1980). Gene flow among populations could also serve to slow or prevent the sorting of particular loci or

combinations of loci within populations. These processes would result in more ISSR loci segregating within populations than markers or arrays of markers that define populations or groups of populations (Archibald et al., 2006). Evolutionary analyses based on dominant markers can be complicated by unreliability (Williams et al. 1993; Smith et al. 1994) and questions of the homology of co-migrating bands, especially as taxonomic distance increases (Rieseberg, 1996; Adams and Rieseberg, 1998). Furthermore, a data analysis within a phylogenetic context is compromised by the anonymous nature of bands, inability to evaluate heterozygosity, and potentially high levels of band polymorphism (Wolfe and Liston, 1998). According to Baldwin et al. (1998), hypervariable DNA markers, as ISSR, are particularly interesting to solve relationships among species originated by recent radiation, as in *Sisyrinchium*. However, in this study, ISSR markers were not able to recover the relationships between species. Already *Cunila* D. Royen ex L., Agostini et al. (2008), the use of ISSR markers, refined the botanical taxonomy formerly accepted in systematics for several species of the genus.

From 24 populations studied here, in five sites of collection, different species occur in sympatry (Figure 1 and 5). Based on the results obtained from STRUCTURE (k=2), there is strong population structure and small gene flow between different species in all analyzed sites. It can indicate that there is an effective barrier to gene flow among species. In addition, these results are in agreement with values of gene flow and population structure to all species of *Sisyrinchium* studied here to the southern Brazil. According to Futuyma (2002), concerning sympatric speciation, a biological barrier prevents the crossing within population without spatial isolation of incipient species. These barriers could be hybridization followed by polyploidization (allopoliploidy), alteration of the mating system of self-incompatible to selfing, chromosomal structural changes which lead to sterility and mutations in genes of great effect on reproductive structures (Judd et al., 1999). In this study, barrier to gene flow among species probably is not related to ploidy level. The sympatric populations ESC 331 and ESC 625 are tetraploid, whereas ESC 497 and ESC 499, ESC 689 and ESC 690 are diploid (Figure 5; Table 1). According to STRUCTURE, these sympatric populations showed no admixture proportion that indicates gene flow. In addition, barrier to gene flow also do not appear be related to breeding system, since as discussed above, the species probably are outcrossing. The existence of two gene pools can occur due to differences in the stages of plants or period of flowering, or hybrid unviable,

which may prevent gene flow between different species even in sympatry.

Polyploidy plays an important role in the diversification of many angiosperm groups and has been common throughout angiosperm evolutionary history (reviewed by Otto and Whitton, 2000; Wendel and Doyle, 2005; Soltis et al., 2007). In Iridaceae, it is estimated that 60% of the species of the Northern Hemisphere and more than 75% of the species of *Sisyrinchium* are polyploid (Goldblatt and Takei, 1997). In some cases, different cytotypes may be reproductively isolated and may actually represent cryptic species (Soltis et al., 2007), while in other cases substantial gene flow or shared ancestral variation among cytotypes could happened (Ramsey et al., 2008). Morphological differences can lead to shifts in pollinators, potentially limiting gene flow between cytotypes (Fehlberg and Ferguson, 2012). Morphological or physiological differences among cytotypes may also lead to the occupation of different ecological niches (Johnson et al., 2003; Suda et al., 2004). Polyploidy, reported in more than 75% of *Sisyrinchium* species (Goldblatt and Takei, 1997), also appears to be related to the complex diversification of *S. micranthum* (Tacuatiá et al., 2012). Chromosome numbers of the species of *Sisyrinchium* belonging to clade V were determined for the first time in this study. These species were analyzed in this study for the first time and revealed the occurrence of populations of *Sisyrinchium* diploids, tetraploids and hexaploids (Table 1; Figure 6). In addition, different ploidy levels are found within species, as *S. commutatum* and *S. luzula.* However, no relationship could be evidenced between population diversity and ploidy level for this species of *Sisyrinchium*.

In conclusion, the populations showed considerable genetic variation within and strong population structuring. The sympatric species presented low gene flow, indicating that there is some barrier to the crossing. Polyploidy seems had contributed to the evolution of these species, as well as to other species of *Sisyrinchium*. Concerning the boundaries of the different species belonging to the clade V of *Sisyrinchium*, it was not possible to discriminate them using ISSR markers, with exception to *S. sellowianum*, although with low support values.

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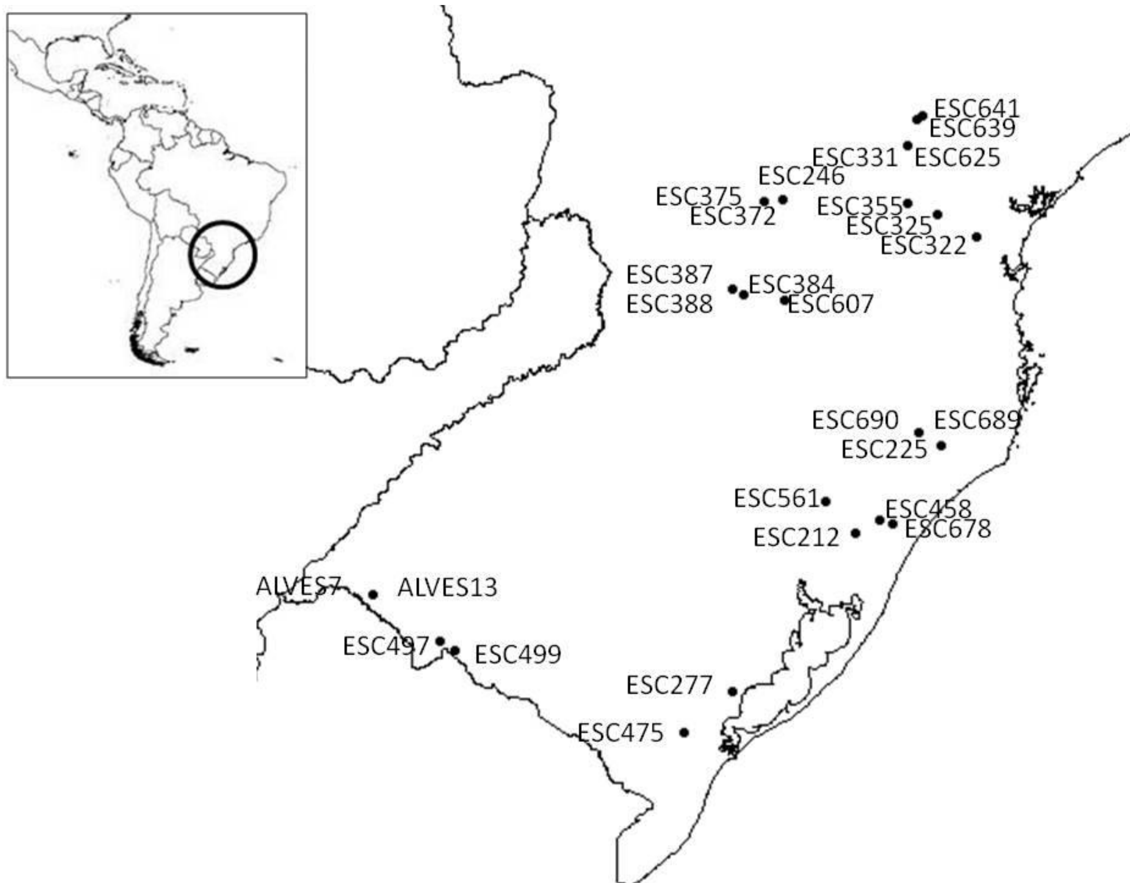

Figure 1 – Distribution of the populations belonging to clade V of *Sisyrinchium*.

Table 1- List of *Sisyrinchium* species analysed, vouchers informations, place and coordinates of collection and chromosome numbers.

Species	Population	No. of individuals	Chromosome number[a]
S. cf. *claritae*[1,2]	ESC 499	28	2n= 2x= 18
S. commutatum[1,2]	ESC 245	14	2n= 2x= 18
S. commutatum[1,2]	ESC 331	30	2n= 4x= 36
S. fiebrigii[1]	ESC 325	1	2n= 6x= 54
S. hoehneii[2]	ESC 355	31	-
S. hoehneii[1,2]	ESC 375	31	2n= 2x= 18
S. luzula[1]	ESC 212	1	2n= 6x= 54
S. luzula[2]	ESC 246	11	-
S. luzula[1]	ESC 322	1	2n= 2x= 18
S. luzula[2]	ESC 384	30	-
S. luzula[2]	ESC 387	31	-
S. luzula[2]	ESC 607	30	-
S. luzula[1,2]	ESC 625	30	2n= 4x= 36
S. luzula[1,2]	ESC 639	9	2n= 2x= 18
S. luzula[2]	ESC 641	30	-
S. aff. *luzula*[1,2]	ESC 678	18	2n= 4x= 36
S. cf. *luzula*[1,2]	ESC 689	31	2n= 2x= 18
S. ostenianum[1] Beauverd.	ESC 475	1	2n= 2x= 18
S. scariosum[1,2]	ESC 277	26	2n= 4x= 36

S. scariosum[2]	Alves13	30	-
S. sellowianum[1,2]	ESC 372	30	2n= 4x= 36
S. sellowianum[2]	ESC 388	32	-
S. sellowianum[1,2]	ESC 458	30	2n= 4x= 36
S. sellowianum[1,2]	ESC 561	33	2n= 4x= 36
S. setaceum[2]	ESC 225	29	-
S. setaceum[1,2]	ESC 690	30	2n= 2x= 18
Sisyrinchium sp. (white)[1,2]	ESC 497	31	2n= 2x= 18
Sisyrinchium sp. (white)[2]	Alves7	10	-

ESC: indicates the collectors Eggers & Souza-Chies. [1]Populations used for determination of ploidy level. [2]Populations used for genetic analyses. [a]Determined in this study.

Table 2 – ISSR primers, total number of markers scored for each primer, and sizes of the amplified fragments.

Primer	Sequence (5’-3’)	Number of recorded markers	Size range (pb)
SV8	$(GA)_8C$	30	300-2080
F4	$(GA)_8YC$	30	200-2080
SP1	$(AG)_8T$	24	250-2080
SP3	$(CT)_8G$	20	350-2080
Total		104	

Table 3 - Percentage of polymorphic loci (P), Shannon's Information Index (*I*), Nei's gene diversity (*H*) and genetic diversity (hs) for each population and species analyzed.

Species	Population	P (%)	*I*	*H*	hs
S. cf. claritae	ESC 499	47.12	0.21	0.14	0.17
S. commutatum	ESC 245	14.42	0.07	0.05	0.08
	ESC 331	25.96	0.09	0.05	0.07
		37.50	0.15	0.10	0.14
S. hoehneii	ESC 355	20.19	0.08	0.05	0.06
	ESC 375	25.00	0.12	0.08	0.10
		43.27	0.20	0.14	0.17
S. luzula	ESC 246	25.96	0.12	0.08	0.11
	ESC 384	33.65	0.15	0.10	0.12
	ESC 387	36.54	0.17	0.11	0.12
	ESC 607	51.92	0.19	0.12	0.14
	ESC 625	55.77	0.25	0.16	0.19
	ESC 639	28.85	0.15	0.10	0.14
	ESC 641	50.96	0.22	0.14	0.16
	ESC 678	26.92	0.12	0.08	0.09
	ESC 689	35.58	0.16	0.11	0.13
		78.85	0.30	0.19	0.16
S. scariosum	ESC 277	30.77	0.14	0.09	0.11
	Alves-13	43.27	0.20	0.14	0.16
		52.88	0.23	0.15	0.23

S. sellowianum	ESC 372	49.04	0.22	0.14	0.17
	ESC 388	47.12	0.16	0.10	0.14
	ESC 458	52.88	0.22	0.14	0.18
	ESC 561	50.96	0.20	0.13	0.15
		75.96	0.29	0.18	0.20
S. setaceum	ESC 225	38.46	0.17	0.11	0.14
	ESC 690	38.36	0.20	0.13	0.14
		58.65	0.27	0.18	0.23
Sisyrinchium sp. (white)	ESC 497	31.73	0.12	0.16	0.11
	Alves7	27.88	0.14	0.10	0.13
		47.12	0.19	0.12	0.23
	Total	97.12	0.33	0.20	0.13

Table 4 – Estimates of population structure for each species analyzed, number of migrants per generation (Nm), *F* statistics (F_{ST}), inbreeding coefficient (f) and θ^B (analogue of F_{ST}).

Species	Nm	F_{ST}	f	θ^B
S. commutatum	0.48	0.71	0.50	0.58
S. hoehneii	0.43	0.73	0.56	0.64
S. luzula	0.70	0.45	0.39	0.45
S. scariosum	1.63	0.40	0.55	0.43
S. sellowianum	1.19	0.38	0.77	0.44
S. setaceum	1.04	0.58	0.53	0.48
Sisyrinchium sp. (white)	0.81	0.53	0.62	0.42
Total	0.54	0.51	0.97	0.51

Table 5 – Analysis of molecular variance (AMOVA) for ISSR data of 533 individuals in 24 populations of eight species of *Sisyrinchium.*

Species	Source of variation	Percentage variation
Total	Among populations	51.33815 [a]
	Within populations	48.66185 [a]
Eight groups (groups corresponding species)	Among groups	7.48514 [a]
	Among populations within groups	44.45564 [a]
	Within populations	48.05923 [a]
S. commutatum	Among populations	71.53 [a]
	Within populations	28.47 [a]
S. hoehneii	Among populations	72.6 [a]
	Within populations	27.4 [a]
S. luzula	Among populations	45.13 [a]
	Within populations	54.87 [a]
S. scariosum	Among populations	40.52 [a]
	Within populations	59.48 [a]
S. sellowianum	Among populations	38.33 [a]
	Within populations	61.67 [a]
S. setaceum	Among populations	58.47 [a]
	Within populations	41.53 [a]
Sisyrinchium sp. (white)	Among populations	52.72 [a]
	Within populations	47.28 [a]

[a] Significance tests after 1000 random permutations.

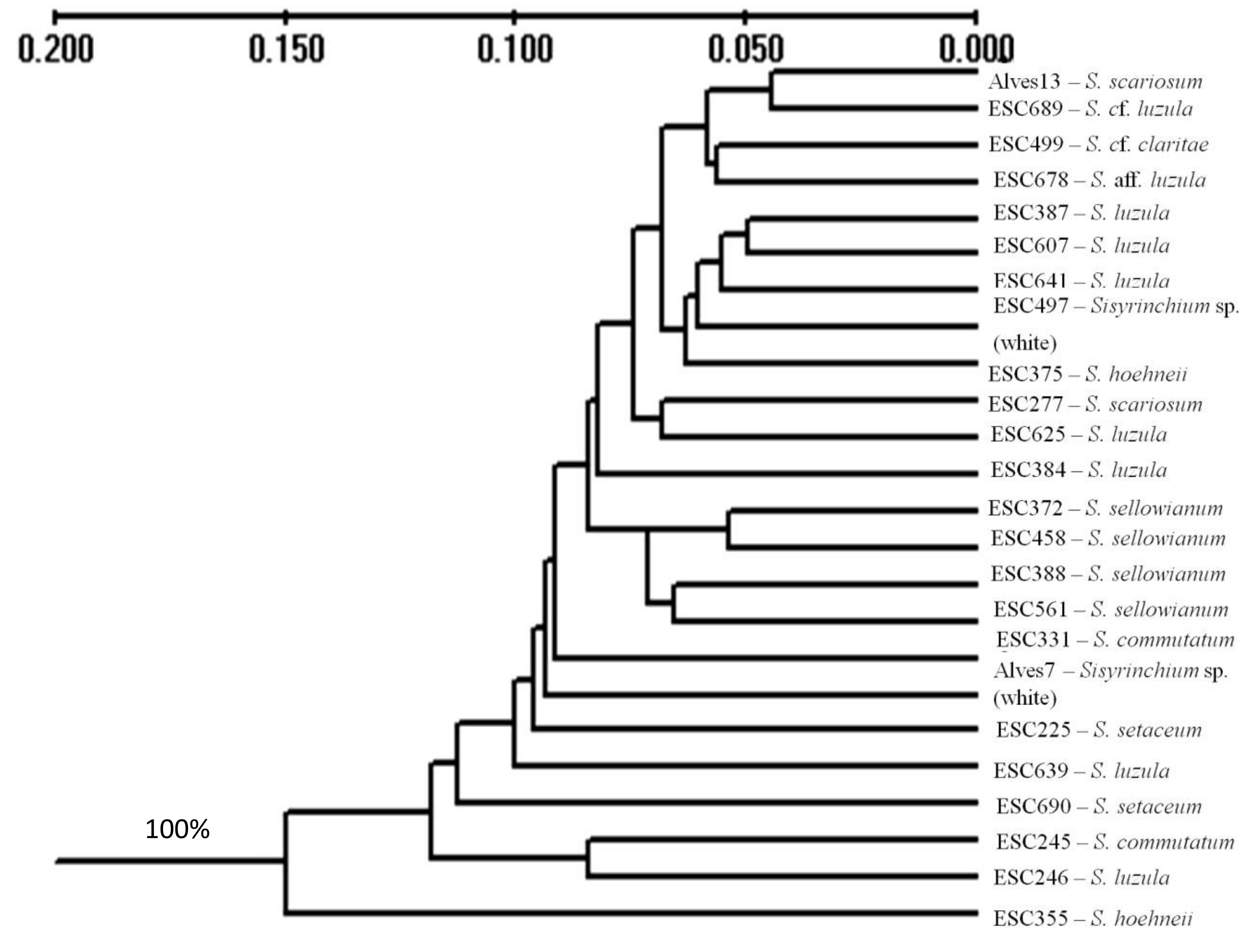

Figure 2 – Unweighted pair-group method arithmetic average (UPGMA) based on Nei (1978) genetic distance (including bootstrap support values in percentages) of populations of *Sisyrinchium*. A scale of genetic distances is provided at the top of the dendrogram.

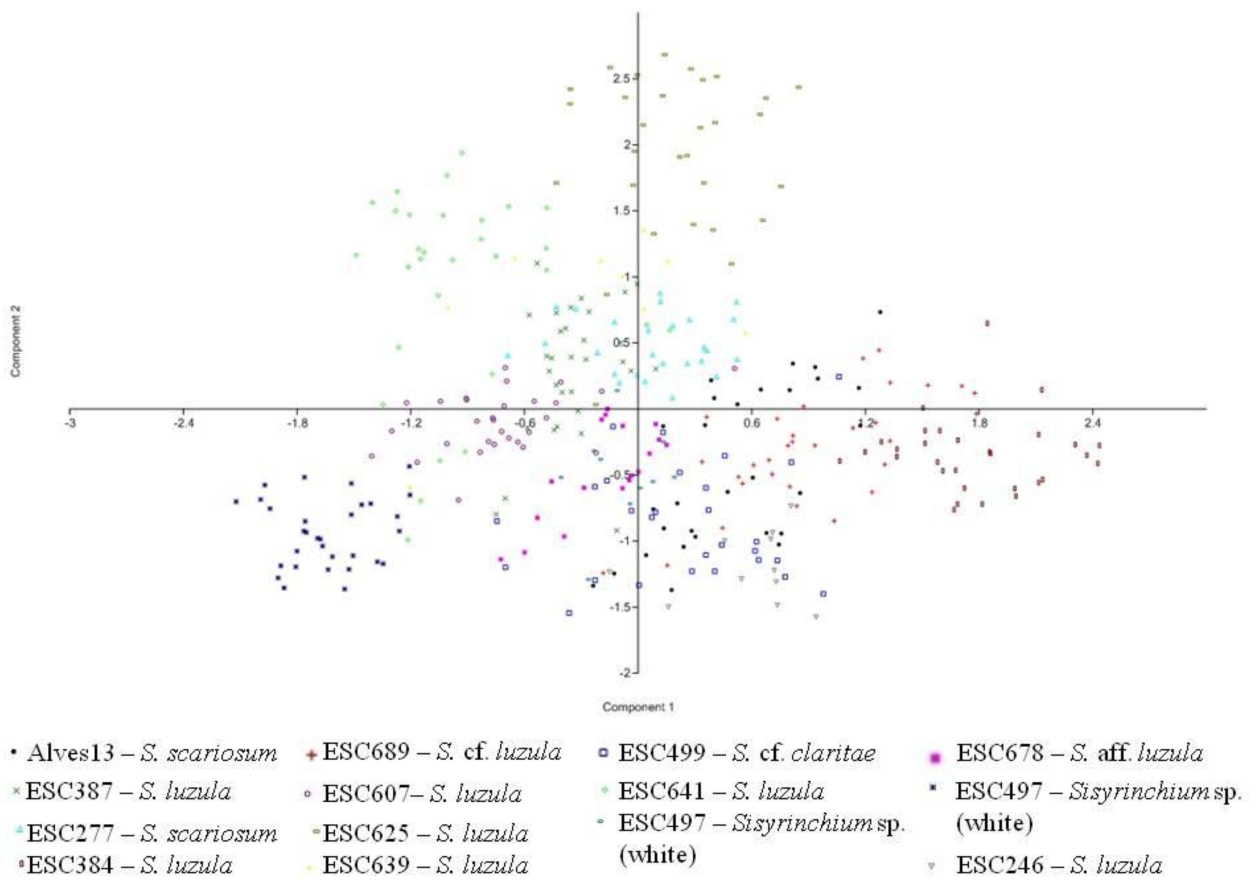

Figure 3 - PCA analysis for the populations of *S. luzula, S.* cf. *claritae* and *S. scariosum*.

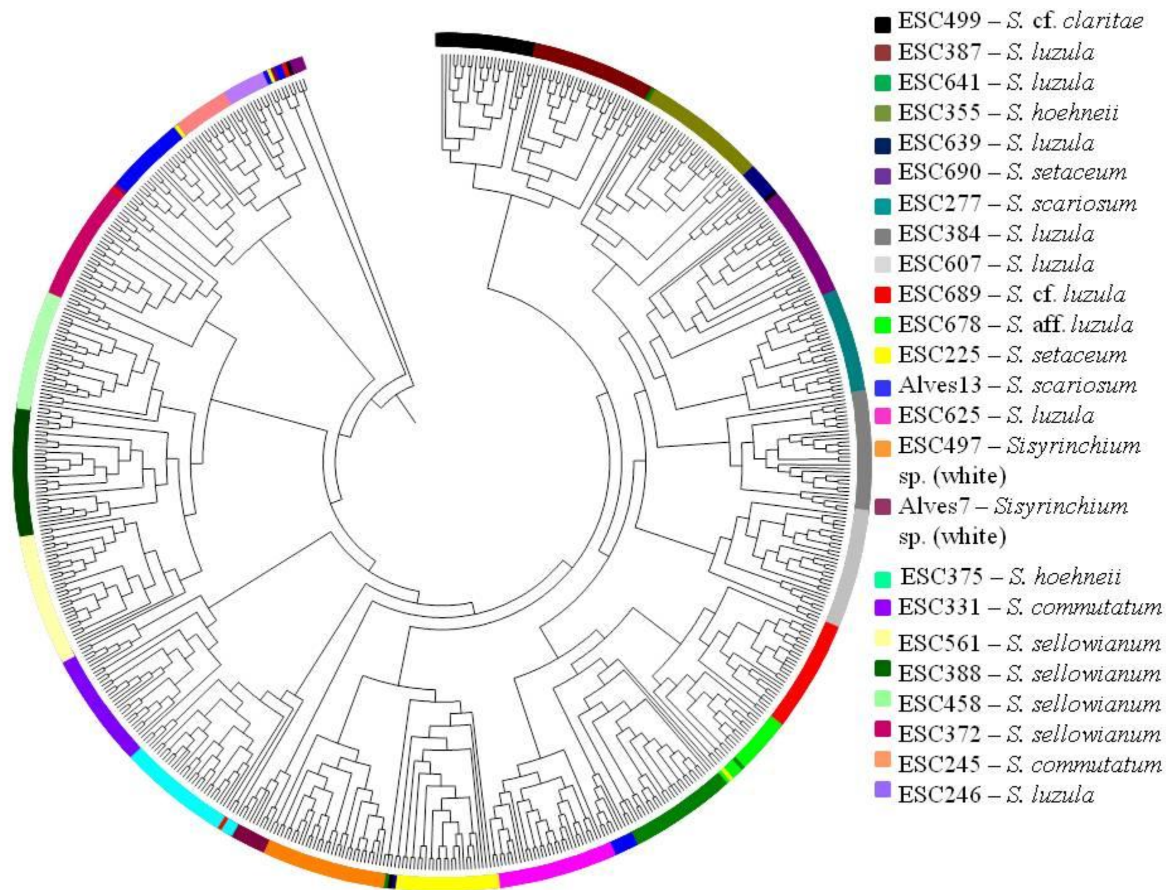

Figure 4 - Dendrogram UPGMA obtained by individuals of the populations of *Sisyrinchium*.

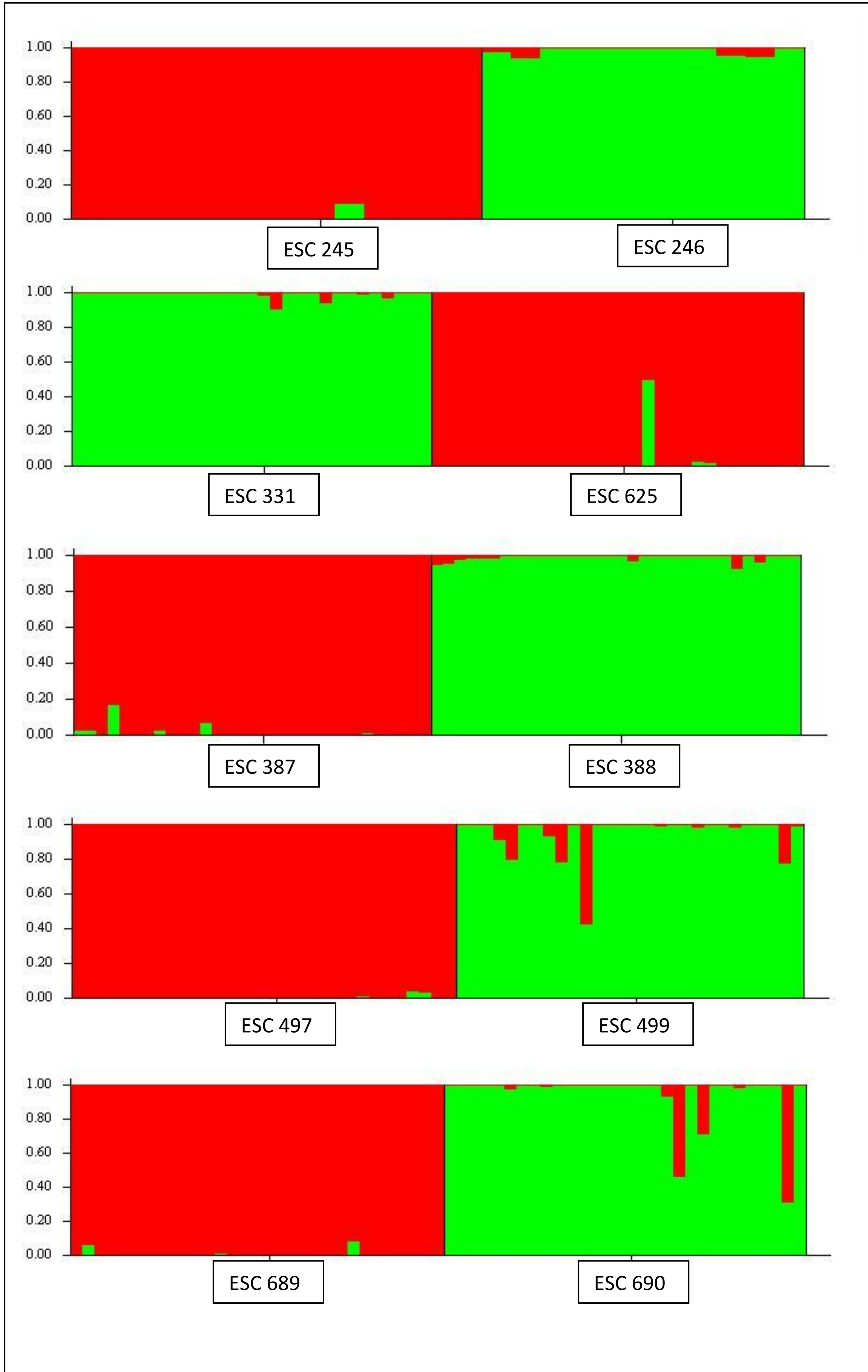
1.00
0.80
0.60
0.40
0.20
0.00
ESC 245
ESC 246
ESC 331
ESC 625
ESC 387
ESC 388
ESC 497
ESC 499
ESC 689
ESC 690

Figure 5 – Bayesian admixture proportions (*Q*) of individual plants of *Sisyrinchium* at each site in which different species occur in sympatry for *k*= 2. Each individual is represented by a single vertical line broken into *k* colored segments, with lengths proportional to each of the *k*-inferred clusters. The most likely number of population (*k*) was estimated with the admixture model and correlated allele frequencies, with no prior information regarding population origin.

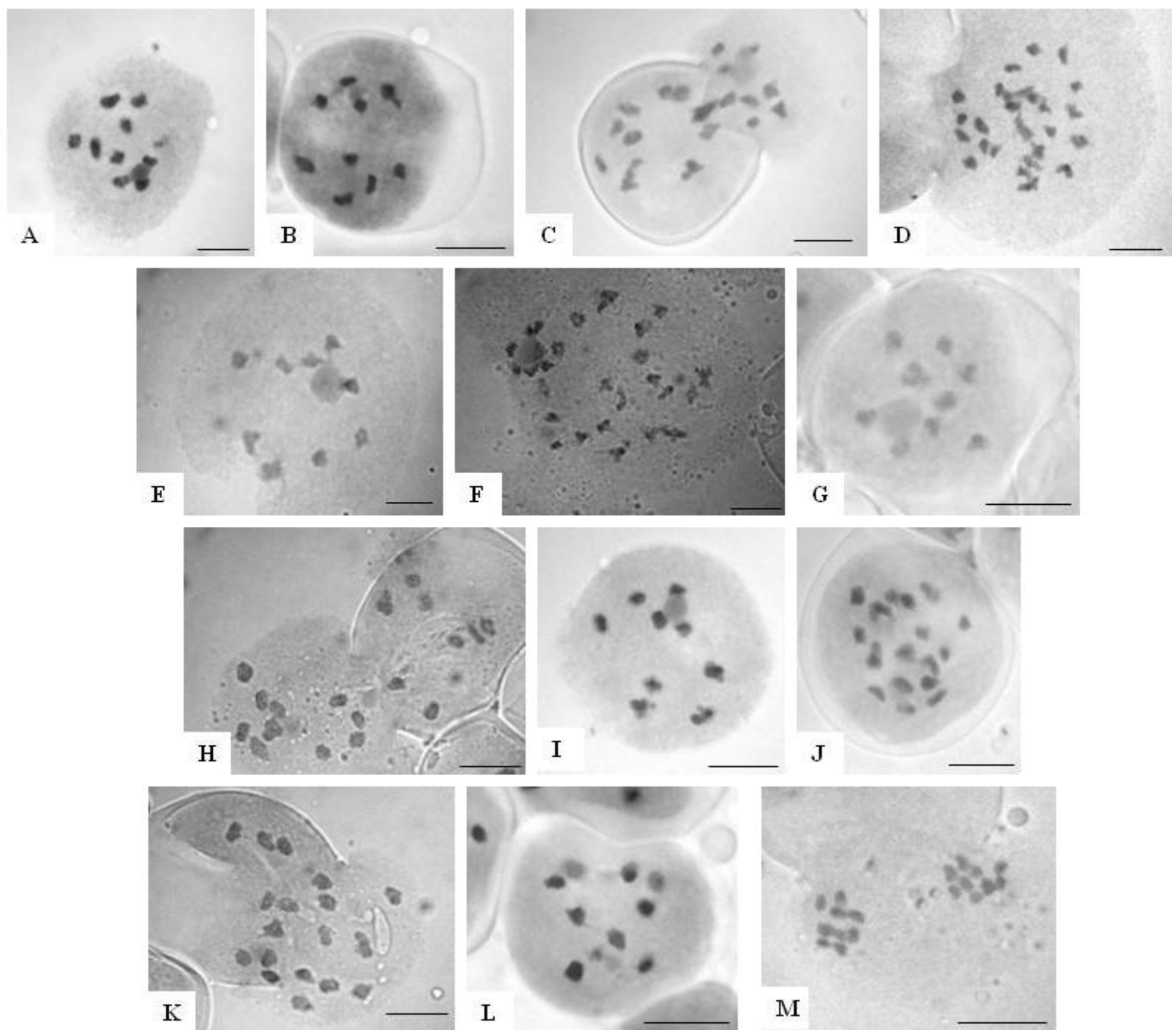

Figure 6 – Meiotic cells of the populations of *Sisyrinchium* species. A) ESC 499 *S.* cf. *claritae*, diakinesis with 9 bivalents chromosomes; B) ESC 245 *S. commutatum*, diakinesis with 9 bivalents chromosomes; C) ESC 331 *S. commutatum* diakinesis with 18 bivalents chromosomes; D) ESC 325 *S. fiebrigii*, diakinesis with 27 bivalents chromosomes; E) ESC 375 *S. hoehneii*, diakinesis with 9 bivalents chromosomes; F) ESC 212 *S. luzula*, diakinesis with 27 bivalents chromosomes; G) ESC 322 *S. luzula*, diakinesis with 9 bivalents chromosomes; H) ESC 678 *S. luzula*, diakinesis with 18 bivalents chromosomes; I) ESC 475 *S. ostenianum*, diakinesis with 9 bivalents chromosomes; J) ESC 277, *S. scariosum*, diakinesis with 18 bivalents chromosomes; K) ESC 561 *S. sellowianum*, diakinesis with 18 bivalents chromosomes; L) ESC 690 *S. setaceum*, diakinesis with 9 bivalents chromosomes; M) ESC 497 *Sisyrinchium* sp. (white), Anaphase I with 9 chromosomes in each pole. Scale= 10 µm.

CAPÍTULO IV

Biological diversity in *Sisyrinchium sellowianum*: different reproductive strategies or a new species?

Sisyrinchium sellowianum apresenta grande diversidade morfológica, sendo classificada em duas categorias morfológicas. Categoria Morfológica I (CM-I – à esquerda) e Categoria Morfológica II (CM-II – à direita).

Fotos: Lilian Eggers e Juliana Fachinetto.

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Biological diversity in *Sisyrinchium sellowianum*: different reproductive strategies or a new species?

Juliana Fachinetto[1], Eliane Kaltchuk-Santos[1], Tatiana Teixeira de Souza-Chies[1,2]*

[1]Departamento de Genética, Universidade Federal do Rio Grande do Sul, CEP 91501-970, Porto Alegre, Rio Grande do Sul, Brasil.

[2]Departamento de Botânica, Universidade Federal do Rio Grande do Sul, CEP 91501-970, Porto Alegre, Rio Grande do Sul, Brasil.

[*]Corresponding Author: Av. Bento Gonçalves, 9500, Universidade Federal do Rio Grande do Sul, Campus do Vale, Departamento de Botânica, Prédio 43433. Bairro Agronomia, Porto Alegre – RS, Brasil CEP: 91501-970. Fone/Fax: (51) 3308 – 7569. E-mail: tatiana.chies@ufrgs.br

Biological diversity in *Sisyrinchium sellowianum*.

Abstract

Remarkable morphological variability on natural populations can be observed in *Sisyrinchium sellowianum* Klatt (Iridaceae family), enabling the classification of the species in two morphological categories. Cytogenetics, reproductive biology and genetic studies were performed aiming to investigate the origin of such variation in *S. sellowianum.* The chromosome number was determined to 15 populations, as well morphology and viability of pollen of each morphological category. Pollinations experiments were performed to determine the reproductive system. A total of 12 populations were analyzed by ISSR-PCR. Genetic diversity (P, *I*, H and Nm), population structure (AMOVA, F_{ST}), Bayesian approach and Mantel Test were calculated. A dendrogram UPGMA based in Nei's genetic distance and Jaccard similarity was constructed. The populations were highly differentiated (F_{ST}= 0.46, θ^B= 0.62). In the morphological category I (MC-I), most of the observed variation was within populations (69%) and 31% among population. On the other hand, in morphological category II (MC-II) 61% of the variation was among populations and 39% within population. The basic chromosome number was x= 9, MC-I presented diploid and tetraploid populations, whereas MC-II had only diploid populations. Different pollen morphologies were observed in each morphological category. Based on pollination experiments, MC-I is out-crossing, whilst MC-II is selfing, not occurring crosses between morphological categories. This study indicates that morphological categories are genetically differentiated and reflect their breeding system, MC-I is alogamous and MC-II is autogamous. Studies based on morphological features are vitally important in order to define if the morphological categories shown here correspond to different species are not.

Keywords: Biological diversity, breeding system, ISSR, morphological category, ploidy level.

Introduction

Sisyrinchium L. (Iridaceae) includes approximately 140 species (Goldblatt *et al*., 2008), extending throughout South, Central and North America. South America is probably the center of origin of this genus (Goldblatt *et al.,* 2008; Chauveau *et al*., 2011). In a recent study, the monophyly of the genus was confirmed and revealed nine major clades weakly connected to the unfinished subdivision established by Ravenna, with some exceptions (Chauveau *et al*., 2011).

Sisyrinchium sellowianum Klatt occurs in Southern Brazil and morphological variation in natural populations was observed. According to our observations, two different morphologies can be found. The main differences are related to the individual size, form of the tepals, herkogamy and particularities in flowering, as floral display and period of flowers anthesis. In Table 1 the main morphological differences for each category are listed.

Great morphological variation can be observed in other species of *Sisyrinchium*. *Sisyrinchium micranthum* Cav. is the most variable species, with morphotypes that can be characterized by a combination of different plant size and habit, flower size and color, and tepal organization. Different morphological categories (CI, CII, and CIII) have been adopted to classify these plants (Tacuatiá *et al*., 2012*a*). In addition, three ploidy levels were found in *S. micranthum* (2n= 16; 2n= 32 and 2n= 48 chromosomes), however, the morphological variation observed for this species is not directly associated with ploidy levels (Tacuatiá *et al*., 2012*a*). Studies of population genetics within this species showed that hexaploid population (2n=48) was genetically less variable than the other populations (Tacuatiá *et al*., 2012*b*). The same authors suggest that this population structure can be related to the selfing reproduction attributed to hexaploids. Other taxa displaying morphological variation and taxonomic problems are *Sisyrinchium palmifolium* L. and *S. vaginatum* Spreng. (Souza-Chies *et al*., 2012).

In face of this panorama to *S. micranthum*, the same situation would be happen in *S. sellowianum*? The morphological categories observed in *S. sellowianum* reflect the different ploidy levels? Are the morphological categories related to reproductive strategy? Are there also differences in population structure of the morphological categories? Is there gene flow between the morphological categories? Thus, the aim of this study was to check if the morphological variation observed in populations of *S. sellowianum* reflects different

reproductive strategies or the existence of a new species or a new variety, by using different approaches. For this, cytogenetics, reproductive biology and genetic studies were performed.

Materials and Methods

Cytogenetic data

Chromosome number was determined in pollen mother cells (PMCs) undergoing meiosis. For this purpose, young inflorescences of 15 populations from *S. sellowianum* were collected and fixed in ethanol: acetic acid (3:1) for 24 h at room temperature and stored at -4°C (Table 2; Figure 1). Plants from each population were classified in two morphological categories according the floral traits described in Table 1. The slides for analysis were prepared through anthers of squashing and staining with 1% propionic carmin on glass slides. Approximately 10 PMCs were analyzed to determine the chromosome number of each accession. A total of 15 accessions, 11 of MC-I and four of MC-II had chromosome number determined (Table 2).

For the analysis of pollen grains morphology and viability, the slides were prepared following Alexander's (1980) method, in which empty unviable pollen grains stain green and full viable pollen grains stain purple. The pollen viability was determined to the different morphological categories and ploidy level of each category, classified as MC-I diploid, MC-I tetraploid and MC-II diploid. At least three populations chosen randomly of each category and ploidy level were used to prepare five slides and 500 pollen grains per slides were analyzed, totalizing 2500 pollen grains per morphological category. Measurements of the polar axis (P) and equatorial diameter (E) were performed in 20 pollen grains per slides for each morphological category (Figure 2).

The classification of pollen grain morphology followed Erdtman (1971). The measures of pollen grains were submitted to the variance analysis (ANOVA) and the averages compared by Tukey test at 5% by BioEstat 5.0, in order to verify if the size of pollen grains varies according to ploidy level.

In three populations used for molecular analysis (ESC 181, ESC 201 and ESC 388) the ploidy levels were inferred by the pollen grains size. Measurements of the polar axis (P) and equatorial diameter (E) were performed in 20 pollen grains per slides totalizing 100 pollen grains per population (Figure 2). These measures were submitted to the variance

analysis (ANOVA) and the averages compared by Tukey test at 5% with the others measures.

Molecular data

A total of 306 samples of *Sisyrinchium sellowianum* from 12 populations in Southern Brazil were collected for inter-simple sequence repeats (ISSR) analysis (Table 2; Figure 1). The populations were classified in two morphological categories according to the floral traits as described in Table 1, being seven populations belonging MC-I and five the MC-II. DNA was isolated from dried leaf samples using a CTAB method modified from Doyle and Doyle (1987). Genetic variation was assessed using seven primers of ISSR (Table 3; Agostini et al., 2008).

PCR was carried out in 25 µL reactions using (depending on the primer): 0-4% DMSO, 1x buffer Taq DNA polymerase (Cenbiot, Rio Grande do Sul, Brazil), 4.0-5.0 mM $MgCl_2$, 0.8 mM dNTP (Invitrogen, São Paulo, Brazil), 0.8 mM of primer, 1U *Taq* DNA polymerase (CenBiot, Rio Grande do Sul, Brazil), 20-30 ng of genomic DNA and completed with water in a termal cycler Veriti 96 Well Thermal Cycle, Applied Biosystems. The thermal cycling program for amplification consisted of initial denaturing at 94 ^{o}C for 5 min, followed by 40 cycles of denaturation at 94 ^{o}C for 1 min, annealing at 48°C for 1 min, extension at 72°C for 2 min 30s, and a final extension step at 72 ^{o}C for 5 min. PCR products were analyzed on 1.5% agarose gels and stained with GelRed (Amicon Corp., Lexington, MA).

Amplified ISSR bands were scored for each individual as binary presence (1) or absence (0) characters. The resulting data matrix was analyzed using POPGENE v. 1.31 (Yeh *et al*., 2000) to estimate four genetic diversity parameters: percentage of polymorphic loci (P), Shannon's Informations Index (*I*), Nei's gene diversity (*H*) (Nei, 1973) and gene flow estimates (Nm) calculated as $Nm = 0.5\ (1\text{-}G_{st})/G_{st}$ (McDermott and McDonald, 1993). These parameters were analyzed for all 12 populations and for each morphological category.

To infer population genetic structure and differentiation among populations, we ran an analysis of molecular variance (AMOVA; Excoffier *et al*., 1992) using the program ARLEQUIM version 3.5 (Excoffier *et al*., 2010). After, populations were grouped according to morphological categories. To evaluate if there is differentiation among populations, F_{ST} values was calculated for all populations and for each morphological

category separately.

An unbiased genetic distance matrix (Nei, 1978) was generated by TFPGA version 1.3 (Tools for Population Genetic Analyses; Miller, 1997) to construct an unweighted pair-group method arithmetic average (UPGMA) topology, which computed 1000 permutations and estimated the confidence limits of the dendrogram. Marker frequencies were estimated based on the Lynch and Milligan (1994) Taylor expansion estimate.

To assess the genetic similarity of individuals across populations, we computed Jaccard similarity coefficients for all pairs of individuals and an UPGMA dendrogram was generated using the program NTSYSpc version 2.1N (Rohlf, 2001).

A Principal Components Analysis (PCA) was performed in Past version 2.15 (Hammer et al., 2001) to recover the non-hierarchical structure in the dataset.

To test the correlation between the unbiased genetic distance matrix generated by TFPGA and geographic distances (in km) among populations, a Mantel Test was performed using GenAlEx population genetics package (Peakall and Smouse, 2006) with 9999 permutations.

A correlation analysis between genetic distance matrix generated by TFPGA and morphological category of the populations were performed in GenAlEx population genetics package (Peakall and Smouse, 2006) with 9999 permutations.

A Bayesian approach proposed by Holsinger *et al.* (2002) was also applied with HICKORY version 1.1, to obtain a more direct estimate F_{ST} from dominant markers, unaffected by Hardy-Weinberg and f assumptions. The *a posteriori* distribution of the θ^B estimator (the estimate F_{ST}) was numerically approximated through a Markov Chain Monte Carlo (MCMC) simulation, and tends to converge to a beta distribution (see Telles *et al.*, 2006). The four models available in the software were tested, i.e., the full model which allows estimating both θ^B and f, models with f or θ^B equal to zero, and, a final model leaving f free to vary so that the sampler does not attempt to estimate f, but chooses f values from its prior distribution, while estimating other parameters during the MCMC run. Model choice was based on the Deviance Information Criterion (DIC; Spiegelhalter *et al.*, 2002). Estimates of genetic diversity (hs; defined as average panmictic heterozygosity) within each population were also calculated.

Reproductive biology data

Pollinations experiments – three populations of *S. sellowianum* were collected, two

of them were classified as MC-I, diploid (ESC 209) and tetraploid (ESC 662), and one MC-II, diploid (LBC 20) (Table 2; Figure 1). About 10 individuals per population were cultivated in vase and maintained in natural conditions. A total of three different controlled pollination treatments were conducted in the populations ESC 662 and LBC 20, to determine the breeding system: (1) SSP – spontaneous self-pollination (flower tagged and isolated); (2) ISP – induced self-pollination (flowers tagged, artificially pollinated with self-pollen and isolated) and (3) ACP – artificial cross-pollination (flowers tagged, artificially pollinated with pollen from flowers of different individuals of the same population and isolated). The flowers were tagged in the stage of floral button before anthesis. The isolation of flowers was done by bagging the entire vase to prevent contact with pollinators. The flowers were not emasculated due to the small size of anthers and fragility of flowers. We also investigated the possibility of occurrence of crossings between the morphological categories, using the populations ESC 209 (MC-I diploid) and LBC 20 (MC-II diploid). For this, one controlled pollination treatment was conducted: (4) – cross-pollination between morphological categories (tagged flowers, stigmas of MC-I artificially pollinated with pollen from flowers of MC-II and isolated). This test was performed only to transfer the pollen grains of MC-II to MC-I, since in MC-II the anthers are very close to the stigma and fruits could be formed by selfing, providing a false positive result. In MC-I, as the contact between anthers and stigmas does not exist, all the fruits will be formed due to the pollen artificially deposited on the stigma, i.e., pollen from the MC-II.

After maturation, the fruits were collected and weighed in precision balance. Seeds were also weighed, obtained the average weight of each seed by weighing five seeds from each fruit. The values of weight were submitted to ANOVA and averages compared by Tukey test 5% by BioEstat 5.0.

Results

Cytological data

Table 2 presents the chromosome number of the populations analyzed. All populations of the two morphological categories have basic number x= 9 chromosomes. The MC-I presented two ploidy levels, diploid and tetraploid, and only diploid populations were evidenced for MC-II (Table 2; Figure 3).

The two morphological categories of *S. sellowianum* presented different pollen grains morphologies. MC-I have pollen grains of oblate spheroidal type whereas MC-II prolate spheroidal (Table 4; Figure 2).

In relation to the size, the pollen grains of MC-II were statistically smaller when compared to the MC-I both diploid and tetraploid (Table 4; Figure 2). In MC-I, the pollen grains differed according to ploidy level, with tetraploids statistically larger than diploids (Table 4). The two morphological categories had high pollen viability, above 87.2% (Table 4).

Three populations had their ploidy level inferred by measures of pollen grains (Table 5). The populations ESC 181 and ESC 201 had pollen grains with similar size to diploid populations, do not differing statistically of values obtained to diploid populations and differing of tetraploids. ESC 388 had pollen grains of similar size to tetraploid population, differing statistically from diploids but do not from tetraploid populations.

Molecular data

A total of 141 *loci* were found across all ISSR markers in the populations, with 139 found in MC-I and 108 in MC-II. The mean number of markers per locus across all *loci* and populations was 20.71 in MC-I with a range of 10-25, and 15.43 in MC-II with a range of 7-22 (Table 3).

Mean heterozygosity was calculated across all polymorphic loci and populations was H= 0.317 in MC-I with a range of 0.118 – 0.213, and 0.249 in MC-II with a range of 0.06 – 0.093 (Table 6). The Bayesian estimates of genetic diversity calculated without assumptions for inbreeding and Hardy-Weinberg equilibrium were similar to the heterozygosity, MC-I (hs= 0.327) and MC-II (0.179; Table 6). Populations were highly differentiated with F_{ST}= 0.46 among all populations combined, 0.307 among MC-I populations, and 0.606 among MC-II populations (Table 7). Bayesian estimates of genetic differentiation (under the full model) were greater than F_{ST} among all populations combined with θ^B= 0.62, θ^B= 0.37 among MC-I populations and θ^B= 0.74 among MC-II populations (Table 7). A comparison of the four models used to calculate genetic diversity and differentiation in HICKORY based on the deviance information criterion (DIC) indicated that the full model, which incorporates inbreeding and population differentiation, was the best fit for the data (DIC= 4212.48).

Results from the AMOVA indicated that most of the observed variation was due to

differences within population (54%) rather than differences among populations (46%) when all populations were analyzed. The same occurred with the populations of the MC-I, the most observed variation was due to differences within population (69%) rather than among populations (31%). However, when only populations of the MC-II were analyzed, most observed variation was due to differences among populations (61%) and 39% within population (Table 7). There was no significant correlation between genetic and geographic distances when all populations were tested (r = 0.11, p = 0.31), and when populations of each morphological category were tested separately (r = 0.147, p = 0.222 for MC-I, and r = 0.305, p = 0.281 for MC-II).

There was a significant correlation between genetic distance and morphological category of populations of *S. sellowianum* (r= 0.71, p= 0.001).

The UPGMA dendrogram generated by NTSYS showed that populations are highly structured, where individuals grouped within their population, in agreement with the high values of F_{ST} observed (Figure 4). In addition, there is no mixture of individuals between the morphological categories. Moreover, it is possible to observe that the individuals of MC-I displayed lower genetic similarity than individuals of MC-II, and among the populations of the MC-I, the individuals belonging to the diploid populations are more similar among themselves than individuals from tetraploid populations (Figure 4). The UPGMA dendrogram by TFPGA evidenced that the populations belonging to MC-I and MC-II were genetically distinct (Figure 5).

These results were highly concordant with the PCA results (Figure 6). In this analysis, the populations of *S. sellowianum* MC-I were highly related between them, while in *S. sellowianum* MC-II, the populations are more distant from each other. The individuals of each population of *S. sellowianum* MC-II are highly related, reflecting strong population structure in this category. In addition, the populations of *S. sellowianum* MC-II formed two groups, ESC337/ LBC20 and ESC686/ JMF3/ JMF4, identical to the dendrogram UPGMA obtained from TFPGA. The two morphological categories are distanced from each other.

Breeding system data

Table 8 present the data obtained from breeding system experiments. Based on our results, MC-I formed fruits only when artificially cross-pollinated, indicating breeding system by allogamy and auto-incompatibility.

MC-II formed fruits in all treatment. These fruits are probably resulted by self-

pollen, since there was no emasculation of flowers. However, the occurrence of a mixed breeding system could not be excluded, since there some fruits were formed when the flowers were artificially cross-pollinated.

When crossing were made between the two morphological categories, only one fruit was formed. However, this fruit aborted on the seventh day after pollination.

The fruits led 21 days for maturation. Average weight of fruits do not differed between the morphological categories and treatments (Table 8). Average weight of seeds of MC-I was statistically largest than MC-II (Table 8).

Discussion

Morphological variation observed in *S. sellowianum* is found in other species of the genus. *Sisyrinchium micranthum* is a good example for this observation. According to Tacuatiá et al. (2012a, b), *S. micranthum* can be classified into three morphological categories. In the morphological categories was observed variation in ploidy level, breeding system and genetic diversity. However, phylogenetic analysis based on DNA sequences the morphological categories were not splitted (Chauveau et al., 2011).

In this study, *S. sellowianum* was classified in two morphological categories and evaluated under cytogenetics, genetics and reproductive aspects. In all aspects the morphological categories showed differences between them. In addition, a phylogenetic study using data from DNA sequences grouped the accessions of *S. sellowianum* MC-I in a strongly supported clade and autapomorphies were observed for these accessions (Fachinetto et al., unpublished).

Polyploidy plays an important role in the diversification of many angiosperm groups and has been common throughout angiosperm evolutionary history (reviewed by Otto and Whitton, 2000; Wendel and Doyle, 2005; Soltis *et al*., 2007). Recent estimates shown that intraspecific variation in ploidy level can be found 12 – 13% of angiosperm species, in which multiple cytotypes occur (Soltis *et al*., 2007; Wood *et al*., 2009). In some cases, different cytotypes may be reproductively isolated and may actually represent cryptic species (Soltis *et al*., 2007), while in other cases there may be substantial gene flow (Ramsey *et al*., 2008).

Polyploidy seems to be an important factor in *Sisyrinchium* evolution, as more than 70% of the studied species are polyploids (Goldblatt and Takei, 1997). According to

Kenton and Heywood (1984), the most frequent basic number for *Sisyrinchium* from the Southern Hemisphere is x= 9, being consistent with the basic number found in this study.

The morphological categories observed for *S. sellowianum* showed variation at the cytogenetic, reproductive and genetic structuring levels. MC-I includes diploid and tetraploid populations, while MC-II includes only diploids populations. The higher number of alleles found for MC-I may be due to the occurrence of tetraploid populations (Table 3).

The presence of polyploid species within a particular group of plants provides an opportunity to test the effects of polyploidy on some morphological traits. One of the most evident effects is a positive relationship between ploidy level and cell size, which has been noted in several groups of plants (Stebbins, 1971; Lewis, 1980). There are also some records of a similar relationship between ploidy level and seed weight and flower size (Chooi, 1971; Solís Neffa, 2000). In this study, pollen grains of the two morphological categories were measured. A positive relation between ploidy level and pollen grain size was found in MC-I (Table 4). Variation in ploidy level has previously been documented for *S. micranthum*, with the existence of three ploidy levels (2n= 16, 2n= 32 and 2n= 48) and a positive relation between ploidy level and pollen grain size was observed (Tacuatiá *et al*., 2012*a*).

In relation to the habitat of different morphological categories, MC-I is more widely distributed, occupying places where the natural vegetation is better preserved. MC-II is more commonly found in anthropized places, such as parks and residential gardens. Based on our sampling, different morphological categories do not occur in sympatry, with the exception of the populations ESC 209 and LBC 20. Only in this place both categories coexist. However, there is a temporal separation in their blooming time, with an overlap period.

Selfing may evolve because it increases seed production when mates or pollinators are scarce, a phenomenon known as reproductive assurance (Darwin, 1876; Baker, 1955; Lloyd, 1965; Inouye *et al*., 1996). The reproductive assurance hypothesis, however, has long been thought to explain shifts towards to selfing, because this strategy is associated with pollinator-poor environments or scenarios where mates are uncommon (Wyatt, 1986; Husband and Barrett, 1991; Kalisz *et al*., 2004). The interactions that result in the mate availability reduction also conduct to the breakdown of self-incompatibility (Young *et al*., 2012). The natural occurrence of the MC-II can explain their breeding system, because is

found in places where probably rates of pollination are reduced due to the lack of pollinators and the reductions in population size by anthropics actions.

In general, selfing is seen as a reproductive strategy that can replace outcrossing whenever the fitness of a selfing morph exceeds that of an outcrossing morph (Lloyd, 1979, 1992). Selection of selfing over outcrossing can potentially occur for an extremely diverse array of reasons (Goodwillie *et al.*, 2005), ranging from its ability to shield individuals and populations from non adaptative gene flow (Antonovics, 1968; Grossenbacher and Whittall, 2011), competitive interactions (Cheptou and Dieckmann, 2002), and antagonists (Koslow and DeAngelis, 2006) or its ability to increase seed production (Darwin, 1876; Lloyd, 1980) or allele transmission in natural populations (Fisher, 1941). Each of the potential benefits of selfing may be countered by inbreeding depression, which has received extensive theoretical and empirical attention in studies of mating-system evolution (Lande and Schemske, 1985; Porcher and Lande, 2005; Byers and Waller, 1999; Keller and Waller, 2002).

Mating systems are primary determinants of the ecological and evolutionary dynamics of plant populations (Barret and Harder, 1996; Charlesworth, 2006). In our study, the morphological categories present different mating systems, MC-I reproduce by allogamy, being auto-incompatible, whereas MC-II is auto-compatible and seems produce their seeds only by selfing. The fruits produced through cross-pollination treatment of CM-II are probably due to residual self-pollen that was deposited on the stigma. In addition, the number of fruits was significantly smaller in cross-pollination treatment than self-pollination (Table 8). Henderson (1976), when studying *Sisyrinchium* species from the Northern Hemisphere, reported a correlation between breeding system and ploidy level. Hand-selfing procedures showed that tetraploids were self-incompatible, whereas most of the higher polyploids were self-fertile.

Transitions in mating systems are often accompanied by characteristic changes in a suite of morphological, physiological and developmental traits. For example, self-fertilizing taxa often have smaller flowers (Wyatt, 1988), higher photosynthetic rates (Mazer *et al.*, 2010) and more rapid development rates (Fenster *et al.*, 1995; Armbruster *et al.*, 2002) when compared with their outcrossing relatives. In *Camissoniopsis cheiranthifolia*, a marked difference in corolla width self-incompatible population exhibited much greater herkogamy, display size and floral longevity. In addition to

reopening for a greater number of days, flowers from self-incompatible populations also stay open much longer during the day compared with flowers from selfing populations (Dart *et al* 2012). In agreement with these, MC-I shown greater floral size, longevity and display compared to the MC-II. Moreover, flowers of MC-I open for three consecutive days, while MC-II only few hours. A positive relationship between flower size and outcrossing rate is well documented for congeneric species or conspecific populations that vary in mating systems (Wyatt, 1984; Ritland & Ritland, 1989; Johnston & Schoen, 1996; Goodwillie, 1999; Armbruster *et al*., 2002; Goodwillie & Ness, 2005). The mating system might also have a direct effect on the number of open flowers per inflorescence and flower longevity (Goodwillie *et al*., 2010).

Flower size differences are easily visible, and it has long been noticed that small-flowered plants that quickly produce many seeds without pollinator visits are often closely related to species with more conspicuous flowers that either seed set only after pollinator visits or are self-incompatible. Such flowers may produce less pollen, or pollen may be less available to pollinators (sometimes, flowers remain closed or only partially open). Smaller flowers will generally also attract fewer pollinator visits (Charlesworth, 2006). In MC-II, although there is little production of flowers, the flowers are small and the opening time is short, there is a high fruit production by selfing. As reviewed by Holsinger (1996), the selective advantage of selfing can be by two ways: selfing increases ovule success when seed production is limited by pollen transfer (reproductive assurance hypothesis or fertility advantage); selfing exhibits an intrinsic advantage in pollen transmission (automatic selection or cost of outcrossing).

The genetic structure of plant populations reflects the interactions of several factors, including the long-term evolutionary history of the species (shifts in distribution, habitat fragmentation and population isolation), genetic drift, mating system, gene flow and selection (Schaal *et al*., 1998). Generally, the breeding system of flowering plant species greatly affects population genetic differentiation (Hamrick and Godt, 1989). Estimates of genetic differentiation between populations for outcrossing species based on AMOVA derived by RAPD markers have usually been <28 %, and for inbred species, estimates of interpopulation genetic variation have usually been >70 % (reviewed in Nybom and Bartish, 2000).

The different morphological categories of *S. sellowianum* are readily distinguished

by all analyses of genetic variation. Each morphological category is separated in UPGMA dendrogram, showing different patterns of genetic differentiation, genetic similarity and distribution of genetic variability. Although these morphological categories can be distinguished genetically, they share a number of loci. This observed genetic differentiation between the morphological categories is most likely due to different breeding system adopted. Populations of the MC-I is characterized by high genetic diversity as indicated by the average gene diversity. The gene diversity and F_{ST} values are similar to values found in outcrossing species (H= 0.317, h_s= 0.327, F_{ST}= 0.307, Nybom, 2004, Tables 6 and 7). Populations of MC-II presents very high values of F_{ST}, similar to the selfing species (F_{ST}= 0.606, Nybom, 2004, Table 7). Populations of each morphological category posses low levels of gene flow or dispersal (Nm= 0.4132, Table 7). AMOVA analysis indicates that the majority of observed differences are due to differences within populations rather than among populations in MC-I, while in MC-II most of the variation is among populations. These results are also in agreement with the differences of the breeding system. Selfing appears to be driven by persistent natural selection in the wild, yet long periods of selfing have negative consequences on the genetic diversity, viability and diversification of plant lineages (Stebbins, 1957; Goldberg *et al.*, 2010). Comparisons of allozyme and nucleotide diversity in closely lineages have repeatedly shown large losses of genetic diversity upon the adoption of selfing (Hamrick and Godt, 1996; Charlesworth, 2003), or high variance in diversity among populations, as would be expected in face of demographic instability (Schoen and Brown, 1991).

Reproductive isolation (or its converse, interbreeding) provides a sharper criterion to objectively define the species category, i.e. that taxa should be accorded the species rank. If two taxa do not interbreed, they must be separated by at least one cladogenetic event (and are separate species). If they interbreed, they most probably have not been separated by a speciation event and are conspecific (secondary introgression is a less likely possibility, but even here it can be argued that the observed taxon represents a fused individual and thus a single species) (Lee, 2003).

In this study, crossings were made between morphological categories. It was verified the possibility of interbreeding in order MC-II to MC-I, due to limitations of floral morphology as previously described. Among the difficulties to perform the crossings should be mentioned the flowering period. Although there is an overlap of the flowering

time for the two morphological categories, the MC-II plants produce fewer flowers with short duration per day. In addition, when the MC-I plants start to flowering, MC-II plants are in the end of the flowering period, producing ever smaller number of flowers, limiting the possibility of interbreeding. In this study, no fruits were formed from this cross, indicating that the two morphological categories have limited ability to gene flow (Table 8). Associated habitat and flowering period, leads to believe that there are important barriers to gene flow.

Separation by habitat is also an important factor for prevent gene flow between plant species (Grant 1981; Futuyma 1998). Gene flow between closely related plant species growing in the same area may be reduced or prevented by temporal differences in flowering phenology or pollinator visitors (Stace 1980; Grant 1981). Temporal separation of flowering and/or pollination time between pairs of closely related species is common and well documented in plants (Harrison 1998).

In conclusion, the two morphological categories described in the present study show differences that go beyond morphology. The differences between the morphological categories are not only due to polyploidy, as in MC-I occur two levels of ploidy (diploid and tetraploid) and do not show marked differences in morphology, whereas MC-II is diploid and has important differences from diploid of MC-I. Our results confirm two mating systems, which is associated with morphological differences in each category. In addition, data from population genetics experiments corroborate the existence of two different reproductive strategies, where the individuals of MC-I have greater intrapopulation variability, while MC-II presents higher interpopulation variability. In relation to the gene flow, our results seem to indicate a reduction or absence between the morphological categories. To define if the two morphological categories studied here represent two distinct species, analyses of DNA sequence are required to support this answer. All results obtained in the present study suggest the existence of two distinct species, which should be subsequently taxonomically revised.

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Table 1 – Comparisons of floral traits and flowering time between the two morphological categories in *Sisyrinchium sellowianum*.

Floral traits	Morphological Category I	Morphological Category II
Flower size	Large	Small
Anther-stigma contact	No	Yes
Floral Display	Aproximatelly 10 flowers per inflorescence	Aproximatelly 3 flowers per inflorescence
Floral Longevity	Three days	Approximately three hours
Initiation of flowering	October	August
End of flowering	December	December

Table 2 – Voucher informations of *Sisyrinchium sellowianum* in Southern Brazil, collection site, number of individuals (N), morphological category (MC) and ploidy level

Population	N	MC	Chromosome Number
ESC 142[a]	1	I	2n= 4x= 36
ESC 165[a]	1	I	2n= 4x= 36
ESC 181[a,c]	11	I	2n= 2x*
ESC 201[a,c]	26	I	2n= 2x*
ESC 209[a,b]	1	I	2n= 2x= 18
ESC 238[a]	1	I	2n= 2x= 18
ESC 241[c]	10	I	-
ESC 372[a,c]	30	I	2n= 4x= 36
ESC 388[a,c]	32	I	2n= 4x*
ESC 458[a,c]	30	I	2n= 4x= 36
ESC 561[a,c]	33	I	2n= 4x= 36
ESC 662[a,b]	1	I	2n= 4x= 36
ESC 337[a,c]	30	II	2n= 2x= 18
ESC 686[a,c]	17	II	2n= 2x= 18
LBC 20[a,b,c]	30	II	2n= 2x= 18
JMF 3[a,c]	30	II	2n= 2x= 18
JMF 4[c]	27	II	-

ESC, LBC and JMF indicates the collectors. [a]Populations used for cytological analysis; [b]Populations used for breeding system analysis; [c]Populations used for ISSR analysis; *ploidy level inferred by measures of pollen grains.

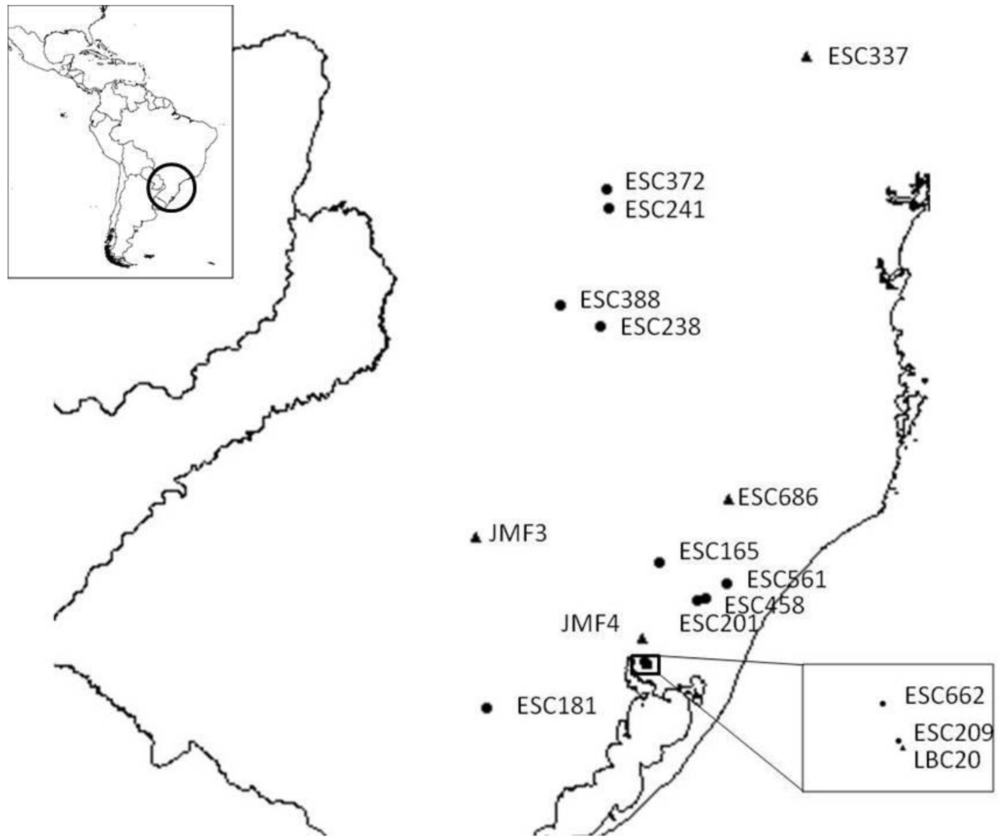

Figure 1 – Distribution of the populations of *Sisyrinchium sellowianum.* Circle indicates *S. sellowianum* MC-I; triangle indicates *S. sellowianum* MC-II.

Table 3 – ISSR primers used for genetic analysis in *Sisyrinchium sellowianum*, sizes of the amplified markers and total number of markers scored for each primer.

Primer	Sequence (5'-3')	Size range (pb)	Number of recorded markers		
			Total	MCI	MCII
SV8	$(GA)_8C$	400-2035	22	22	13
F3	$(AG)_8YC$	250-2010	24	24	22
P2	$(GA)_8T$	350-2080	21	21	19
F4	$(GA)_8YC$	300-2080	25	25	14
SP1	$(AG)_8T$	300-2080	22	22	19
SP3	$(CT)_8G$	350-2080	15	15	14
SP6	$(GACA)_4$	450-1500	12	10	7
Total			141	139	108

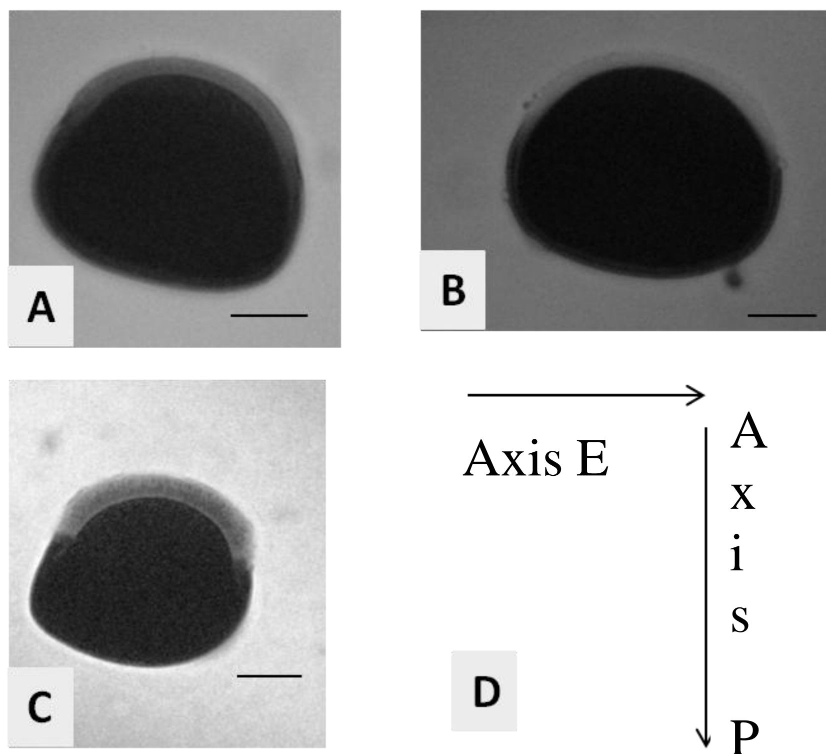

Figure 2 – Pollen grains of *S. sellowianum*. (A) ESC 238, *S. sellowianum* MC-I, diploid, pollen oblate spheroidal. (B) ESC 165, *S. sellowianum* MC-I, tetraploid, pollen oblate spheroidal. (C) ESC 337, *S. sellowianum* MC-II, diploide, pollen prolate spheroidal. (D) Indication of polar axis (P) and equatorial axis (E) of pollen grains. Scale 10 µm.

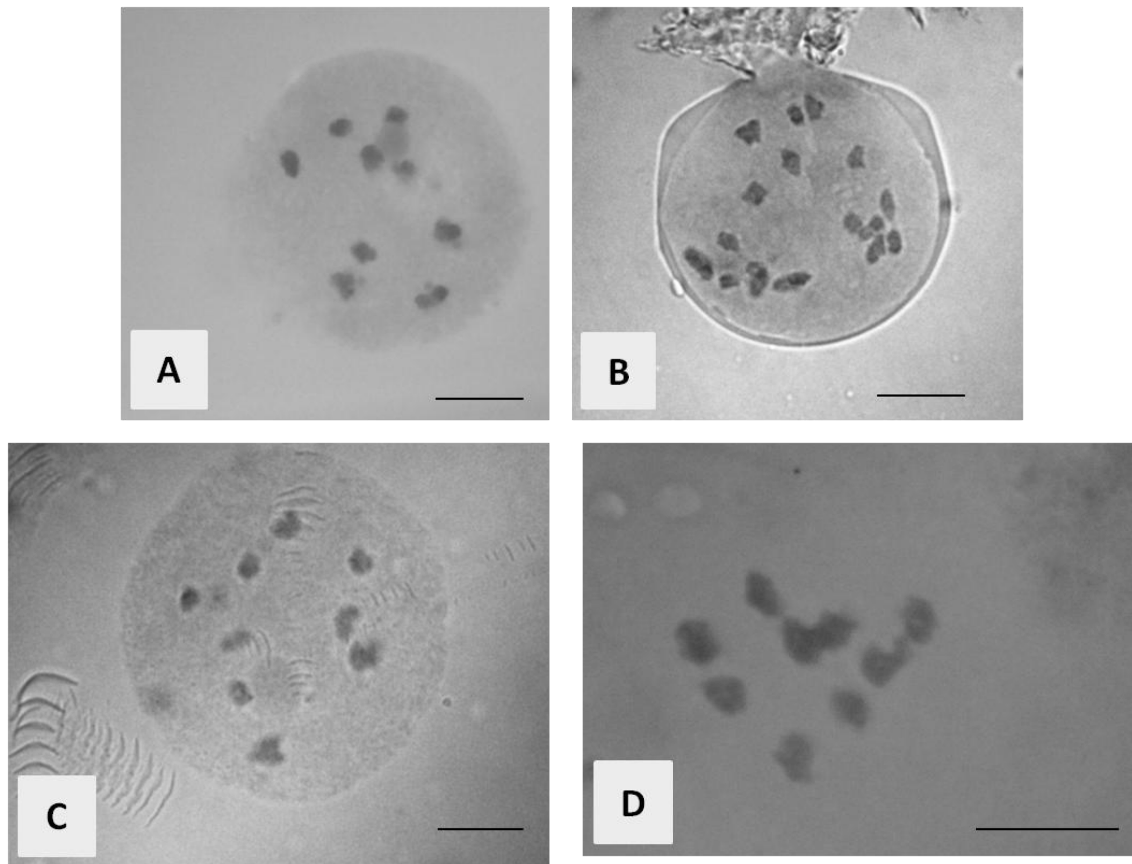

Figure 3 – Meiotic cells of *S. sellowianum*. (A-B) *S. sellowianum* MC-I. (A) ESC 209, diploid. (B) ESC 561, tetraploid. (C-D) *S. sellowianum* MC-II. (C) ESC 337, diploid. (D) LBC 20, diploid. Scale 10 µm.

Table 4 – Pollen morphology in according to Erdtman (1971) and viability of pollen grains in *Sisyrinchium sellowianum*.

Morphological Category (ploidy level)	Axis Polar (P) (µm)	Axis Equatorial (E) (µm)	P/E	Morphology	Viability (%)
MC-II (2x)	26.275a	26.35a	1.00	Prolate spheroidal	98.12
MC-I (2x)	25.525b	29.2b	0.87	Oblate spheroidal	87.2
MC-I (4x)	27.375c	31.15c	0.88	Oblate spheroidal	90.76

Averages followed by the same letter do not differ statistically by Tukey test (p= 0.05).

Table 5 – Ploidy level inferred from measures of pollen grains.

Population/ Morphological category	Axis Polar (P) (µm)	Axis Equatorial (E) (µm)	Ploidy level inferred
ESC 181-MC-I	25.75	29.78	2x
ESC 201-MC-I	25.42	28.72	2x
ESC 388-MC-I	27.2	31.37	4x

Table 6 – Genetic diversity in populations of *S. sellowianum.* Percentage of polymorphic loci (P), Shannon's Information Index (*I*), Nei's gene diversity (*H*) and genetic diversity (hs).

Population	H	I	P (%)	hs
ESC 181	0.118	0.182	39.72	0.151
ESC 201	0.136	0.213	50.35	0.160
ESC 241	0.139	0.216	47.52	0.180
ESC 372	0.213	0.326	68.79	0.252
ESC 388	0.170	0.272	68.79	0.216
ESC 458	0.184	0.288	68.79	0.228
ESC 561	0.162	0.257	65.96	0.194
ESC 337	0.082	0.127	28.37	0.097
ESC 686	0.066	0.102	21.99	0.082
LBC 20	0.093	0.145	36.17	0.078
JMF 3	0.087	0.133	27.66	0.081
JMF 4	0.060	0.017	18.44	0.060
Média	0.125	0.190	45.21	0.148
MC- I	0.317	0.250	58.56	0.327
MC-II	0.249	0.105	26.51	0.179

Table 7 – Gene flow estimates (Nm), F_{ST}, *f*, θ^B (analogue of F_{ST}) and distribution of variability for all population analyzed for each morphological category in *S. sellowianum.*

Morphological category	Number of migrants (Nm)	F_{ST} (p)	θ^B	*f*	Intrapopulation variability	Interpopulation variability
All populations	0.4132	0.46 (0.000)	0.62	0.96	54%	46%
MC-I	0.9342	0.307 (0.000)	0.37	0.25	69%	31%
MC-II	0.2285	0.606 (0.000)	0.74	0.41	39%	61%

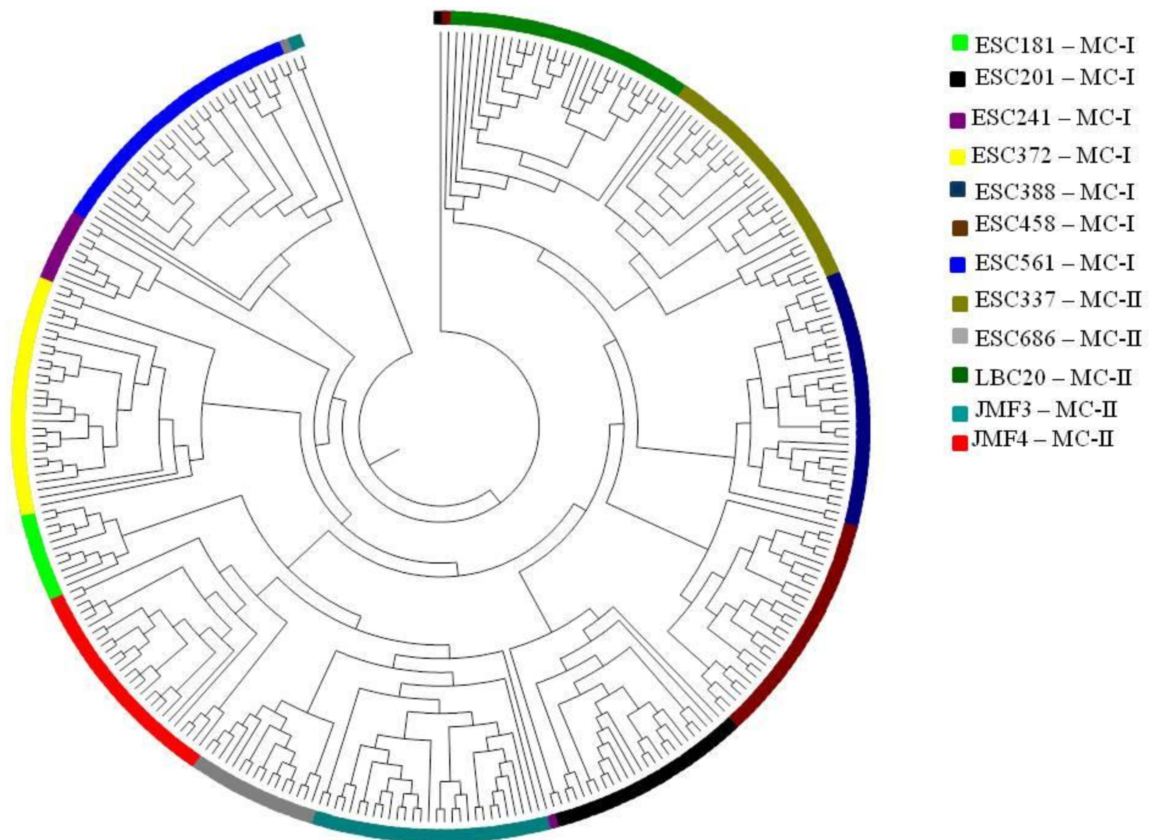

Figure 4 – Dendrogram UPGMA obtained by individuals of the populations of *Sisyrinchium sellowianum*.

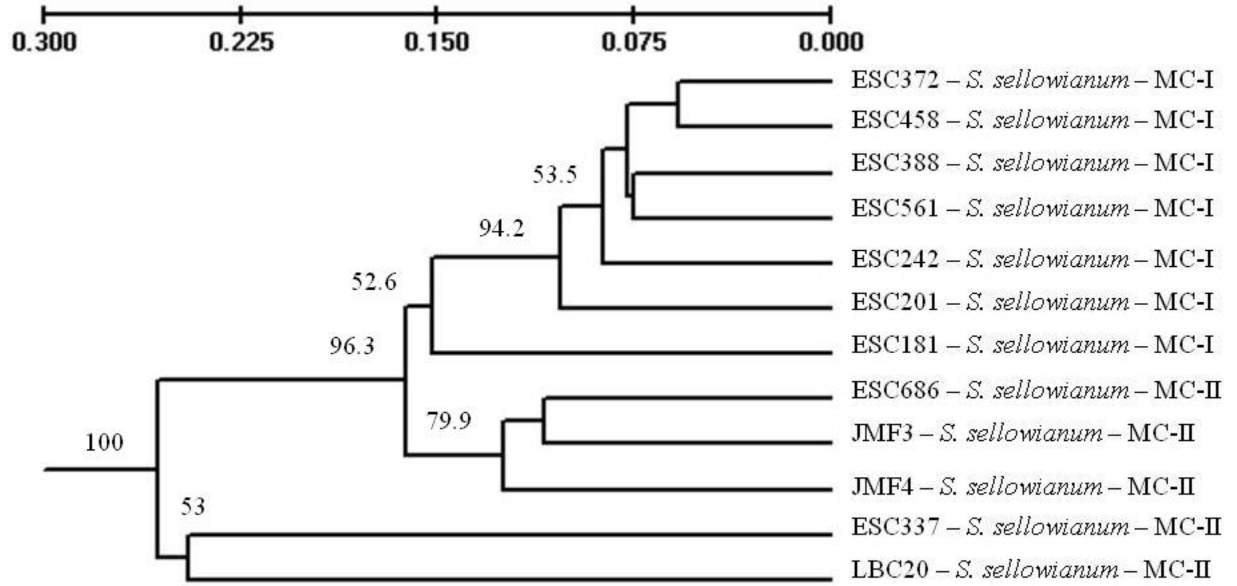

Figure 5 - UPGMA dendrogram based in the Nei's genetic distance. Bootstrap values >50% are above the branches.

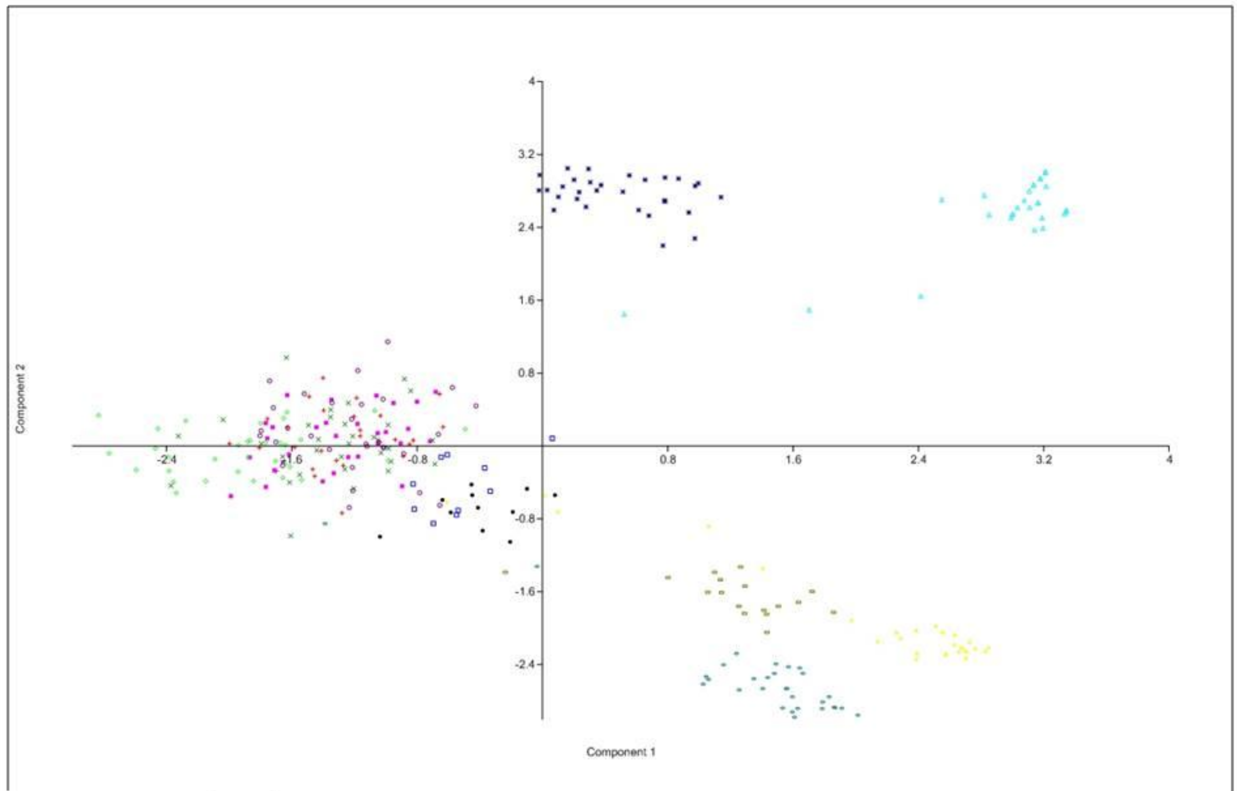

Figure 6 – PCA analysis for the populations of *S. sellowianum*.

Table 8 – Number of tagged flowers, formed fruits, average weight of fruits and seeds of each treatment to determine of breeding system of *Sisyrinchium sellowianum*.

Morphological Category	Treatment	Number of flowers tagged	Number of fruits formed	Percentage of fruits formed (%)	Average weight of fruits (mg)	Average weight of by seeds (mg)
MC-I	SSP	50	-	-	-	-
	ISP	50	-	-	-	-
	ACP	50	38	76	19.1	0.80*
MC-II	SSP	50	37	74	15.7	0.32
	ISP	37	26	70.3	16.0	0.32
	ACP	39	15	38.46	15.0	0.24
MC-I x MC-II	ACP	30	1(aborted)	3.33	-	-

SSP- spontaneous self-polination; ISP – induced self-pollination; ACP – artificial cross-pollination.* Significance Tukey test $p<0.05$.

CAPÍTULO V

DISCUSSÃO FINAL

A presente tese expõe dados inéditos quanto à diversidade genética, citogenética e filogenia das espécies pertencentes ao clado V de *Sisyrinchium.* Além disso, um maior enfoque foi dado à espécie *S. sellowianum* a fim de caracterizar as duas categorias morfológicas observadas. Este trabalho encontra-se inserido em um amplo projeto que visa compreender as interações evolutivas que resultaram nos padrões de diversidade observados em Iridaceae.

Sisyrinchium é um grande gênero de Iridaceae, apresentando o maior número de espécies da família para o sul do Brasil. Recentemente, a filogenia do gênero vem sendo investigada, cabendo ressaltar os estudos de Chauveau et al. (2011) e Karst & Wilson (2012). Esta tese está embasada na filogenia obtida por Chauveau et al. (2011), por considerar uma maior amostragem do gênero e de todas as seções morfológicas descritas por Ravenna (2000, 2001a, 2001b, 2002, 2003a, 2003b).

De acordo com a reconstrução filogenética proposta por Chauveau et al. (2011), *Sisyrinchium* é um gênero monofilético que encontra-se separado em nove clados fortemente a moderadamente suportados, os quais pouco têm em comum com as classificações morfológicas propostas anteriormente (Ravenna 2000, 2001a, 2001b, 2002, 2003a, 2003b).

Outro aspecto importante ressaltado na filogenia inferida por Chauveau et al. (2011), bem como por outros especialistas em Iridaceae (Goldblatt et al., 1998; Goldblatt & Manning, 2008) é a presença de tricomas glandulares produtores de óleos florais e sua localização nas espécies, os quais podem estar localizados na coluna estaminal das flores e/ou na face adaxial das tépalas. Estima- se que cerca de 35% das espécies de *Sisyrinchium* apresentem este tipo de recompensa floral. Óleos florais é um tipo de recompensa a polinizadores bastante restrita entre as famílias botânicas atualmente reconhecidas, tendo sido discutida em cerca de 1.500 a 1.800 espécies distribuídas em onze famílias botânicas (Renner & Schaefer, 2010).

A filogenia inferida para as espécies do clado V, apresentada no **capítulo 2**, reforça a monofilia para o clado, com altos valores de suporte. Além disso, algumas

espécies formaram grupos bem suportados, enquanto outras não. Numa tentativa de melhorar o entendimento sobre as relações entre as espécies de *Sisyrinchium* pertencentes ao clado V, uma abordagem com marcadores ISSR foi feita, já que este marcador é de evolução mais rápida que as sequencias de DNA, o que poderia ser mais efetivo no caso de espécies de evolução recente como as analisadas aqui.

No **capítulo 3**, populações de diferentes espécies de *Sisyrinchium* pertencentes ao clado V foram caracterizadas por marcadores ISSR, além de uma análise citogenética para determinar o nível de ploidia das espécies. Quanto ao número cromossômico básico, as espécies estudadas apresentaram x = 9 cromossomos. Variação no nível de ploidia intra-específico foi observado, com populações diploides, tetraploides e hexaploides. Estes resultados estão em acordo com os dados obtidos para o gênero, que tem mostrado uma forte tendência à poliploidia (Goldblatt & Takei, 1997). As espécies de *Sisyrinchium* avaliadas neste estudo mostraram um alto grau de estruturação populacional.

Em *Sisyrinchium,* os sistemas de cruzamento podem variar de completa autoincompatibilidade a completa autocompatibilidade (Ingram, 1967; Henderson, 1976; Cholewa & Henderson, 1984). A distribuição da variação genética inter e intra-populacional pode fornecer indícios quanto ao sistema de cruzamento das espécies. *Sisyrinchium commutatum*, *S. hoheneii, S. setaceum* e *Sisyrinchium* sp. (white) apresentam maior variação inter-populacional, o que pode caracterizar as espécies como autocompatíveis. Para as demais espécies, a distribuição da variação genética sugere autoincompatibilidade. O dendrograma UPGMA baseado na distância genética de Nei (1978) não foi capaz de agrupar as populações de acordo com as espécies. A única espécie que apresentou um agrupamento foi *S. sellowianum*, no entanto com baixos valores de suporte. Como há registros na literatura de híbridos interespecíficos em *Sisyrinchium* (Henderson, 1976; Cholewa & Henderson, 1984; Goldblatt & Takei, 1997), as espécies pertencentes ao clado V, que ocorrem em simpatria, foram analisadas para investigar possível fluxo gênico entre diferentes espécies. A proporção de mistura entre as espécies foi muito baixa, sendo provavelmente, retenção de polimorfismo ancestral ou tempo de divergência muito recente.

No **capítulo 4** foi investigada a diversidade biológica existente nas populações de *S. sellowianum*. De acordo com as observações durante as coletas, *S. sellowianum* apresentou uma diversidade morfológica considerável, sendo possível a classificação da

espécie em duas categorias morfológicas. *S. sellowianum* CM-I (CM= categoria morfológica) é caracterizada por plantas e flores maiores que *S. sellowianum* CM-II. Um maior número de flores abertas ao mesmo tempo (exibição floral) e uma maior longevidade floral também caracteriza a CM-I quando em comparação com CM-II. Também foi observada uma separação por habitat das duas categorias morfológicas, onde CM-I ocorre em ambientes naturais, enquanto CM-II ocorre em ambientes antropizados. Quanto ao período de florescimento, CM-II é mais precoce que CM-I, entretanto, ocorre um período de sobreposição, possibilitando cruzamentos entre as categorias quando em simpatria. No entanto, a principal diferença floral entre as categorias morfológicas é a hercogamia, CM-I não apresenta contato entre anteras e estigmas, enquanto a CM-II apresenta anteras e estigmas no mesmo nível, proporcionando íntimo contato entre as partes reprodutivas.

As categorias morfológicas apresentaram índices de diversidade genética consideravelmente diferente, sendo a CM-I com maiores valores de diversidade (H, I, P e hs, ver tabela 4) quando comparado com CM-II. Os valores de F_{ST} indicam forte estruturação populacional para as duas categorias morfológicas. Diferenças também quanto ao numero de migrantes e na distribuição da variação genética foram observadas entre as duas categorias morfológicas.

De acordo com o dendrograma UPGMA baseado na distância genética de Nei (1978) as categorias morfológicas encontram-se separadas entre si, com consideráveis valores de suporte.

Em relação às análises citogenéticas, as categorias morfológicas também apresentaram diferenças. CM-I é constituída de populações diploides e tetraploides, enquanto CM-II apenas de populações diploides. A morfologia polínica também diferiu, CM-I possui pólen do tipo oblado esferoidal, enquanto CM-II prolado esferoidal.

Os estudos para determinar o sistema reprodutivo das categorias morfológicas revelaram que CM-I é alógama e a CM-II é autógama. Este resultado está de acordo com os valores obtidos pela análise por marcadores moleculares discutidos anteriormente. MC-I é alógama, então apresenta maiores níveis de diversidade genética, menor estruturação genética das populações, pois maior variação genética ocorre dentro das populações que entre as populações e um maior número de migrantes. Já MC-II é autógama, sendo esperados menores níveis de diversidade genética, maior estruturação populacional, pois

uma maior variação genética ocorre entre as populações, bem como um menor número de migrantes.

Em *S. sellowianum* MC-II, pode-se imaginar diversos cenários que justifiquem sua baixa diversidade genética: reprodução assexual, autofecundação, baixa atração de polinizadores devido ao reduzido tamanho floral e exposição floral, além de baixa disponibilidade de polinizadores devido ao habitat que apresenta interação antrópica.

De acordo com a literatura, auto-polinização é altamente provável quando a antera está ao mesmo nível que o estigma, enquanto polinização cruzada pode ocorrer quando a coluna estaminal é mais curta que o estigma (Ingram, 1968).

Geralmente, espera-se que as plantas poliploides apresentem taxas maiores de autofecundação do que seus progenitores diploides (Stebbins, 1950). Estudos realizados por Ingram (1967) e Henderson (1976) em espécies de *Sisyrinchium* do Hemisfério Norte reportam uma correlação entre sistema de cruzamento e nível de ploidia onde autofertilização é frequente nos mais altos níveis de ploidia. Em *S. micrathum,* as populações diploides e tetraploides são auto-incompativeis, enquanto as populações hexaploides são autoférteis (Tacuatiá et al., 2012b). As populações hexaploides de *S. micranthum* apresentam as mesmas características que *S. sellowianum* CM-I, com plantas e flores menores, além de contato entre anteras e estigmas. Em *S. sellowianum*, no entanto, a autofecundação ocorre na CM-II, a qual é diploide.

Quando realizados os cruzamentos entre as categorias morfológicas, houve a formação de apenas um fruto que não chegou a desenvolver-se até a maturação, e o mesmo não apresentou a formação de sementes. Estes resultados reforçam que existem barreiras reprodutivas entre as duas categorias, dificultando o fluxo gênico.

Quanto à morfologia, é esperada uma relação entre o porte e o nível de ploidia dos indivíduos já que poliploides, geralmente, apresentam em sua morfologia o efeito "*giga"*, o que resulta em flores, grãos de pólen e sementes maiores (Ramsey & Schemske, 2002). Isso foi observado quando analisado o tamanho dos grãos de pólen de *S. sellowianum* CM-I, os grãos de pólen dos tetraploides foram significativamente maiores que os diploides. Em *S. micranthum,* os grãos de pólen dos hexaploides também refletiram o efeito do nível de ploidia, apresentando tamanho significativamente maior.

Os resultados obtidos no capítulo 2 estão em acordo com os obtidos no **capítulo 3**, onde os marcadores ISSR também não foram capazes de agrupar as populações de

acordo com a espécie. Ambos os estudos indicam que marcadores moleculares sozinhos não foram capazes de delimitar as espécies deste clado. Estudos que utilizam marcadores moleculares em associação a outras abordagens, como morfológicas, reprodutivas, citológicas, podem ser mais informativos e resolverem melhor a filogenia e responder outras questões evolutivas referentes a este e aos demais clados de *Sisyrinchium*.

Na filogenia inferida no **capítulo 3**, os acessos de *S. sellowianum* CM-II formaram um grupo fortemente suportado, o que, somado aos resultados obtidos no **capítulo 4** reforça a hipótese de que esta possa ser uma espécie ou subespécie distinta de *S. sellowianum* CM-I.

Todos os resultados obtidos nesta tese podem contribuir para o entendimento e esclarecimento de diversas questões sobre as espécies estudadas aqui e o gênero *Sisyrinchium* como um todo. Os padrões de diversidade e as questões levantadas para diversas espécies de *Sisyrinchium* são similares, o que aumenta a importância dos estudos realizados com o clado V neste trabalho. Outras espécies de *Sisyrinchium* merecem ser analisadas do ponto de vista morfológico, como as espécies não determinadas, mencionadas no **capítulo 2,** pois a possibilidade de espécies novas neste gênero é elevada.

ANEXOS

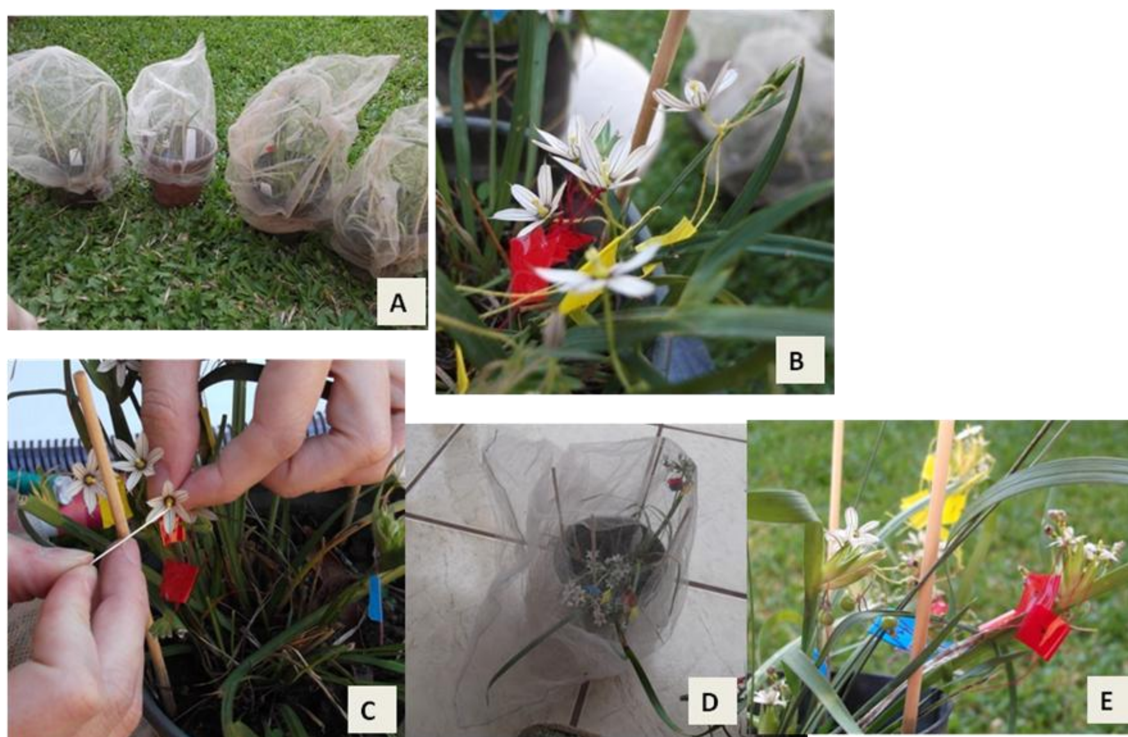

Figura A1 – Procedimento adotado para os experimentos de polinização manual (autopolinização e polinização cruzada) em *S. sellowianum* CM-I. (A) Plantas de *S. sellowianum* CM-I isoladas por sacos de tecido. (B) Planta de *S. sellowianum* CM-I contendo dois tratamentos identificados: fita amarela: autopolinização espontânea; fita vermelha: autopolinização manual. (C) Procedimento de autopolinização manual. (D) Planta isolada com flores abertas contendo os diferentes tratamentos de polinização. (E) Formação dos frutos nas flores de polinização cruzada (fita azul). Fotos: Juliana Fachinetto.

Figura A2 - Procedimento adotado para os experimentos de polinização manual (autopolinização e polinização cruzada) em *S. sellowianum* CM-II. (A) Planta de *S. sellowianum* CM-II isoladas por sacos de tecido. (B) Planta de *S. sellowianum* CM-II contendo tratamento de polinização cruzada identificado com fita azul. (C) Procedimento para a polinização onde o estigma da flor era exposto manualmente. (D) Formação dos frutos nas flores de autopolinização (fita amarela). Fotos: Juliana Fachinetto.

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