UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL

Caracterização Genética da espécie *Sisyrinchium palmifolium* L. (Iridaceae) e espécies relacionadas na região sul do Brasil.

***Sisyrinchium palmifolium* L.**

Rogéria Beatriz Miz

Tese de Doutorado

Porto Alegre

2013

UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL

Instituto de Biociências

Programa de Pós Graduação em Genética e Biologia Molecular

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Tese submetida ao Programa de Pós-Graduação em Genética e Biologia Molecular da Universidade Federal do Rio Grande do Sul como requisito parcial para a obtenção do Título de Doutor em Ciências (Genética e Biologia Molecular).

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Porto Alegre, outubro de 2013

O presente trabalho foi executado no Laboratório de Sistemática Molecular do departamento de Botânica da Universidade Federal do Rio Grande do Sul.

As fontes financiadoras foram:

- Conselho Nacional de Desenvolvimento Científico e Tecnológico (**CNPq**).

-Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (**CAPES/COFECUB).**

- Fundação de Amparo a Pesquisa do Estado do Rio Grande do Sul **(FAPERGS).**

Que delícia é viver e realizar os nossos sonhos. Recomendo a todos!

Dedico esta tese à minha família. Meu filho Eric que vivenciou desde o início comigo a realização de um sonho, muitas vezes, sendo privado de atenção, e sempre retornando com palavras de amor. Ao meu marido Ivan pelo amor, carinho e apoio em todas as horas. A minha mãe amada que sempre me incentivou e me ensinou muito com sua paciência e dedicação. Ao mais novo membro da família Leonardo Miz Schüler que se encontra acolhido em meu ventre e que tem suportado firme e forte a tensão dos últimos meses de tese. Amo vocês!

AGRADECIMENTOS

À minha orientadora Tatiana Teixeira de Souza Chies que desde o meu primeiro semestre de faculdade me acolheu em seu laboratório e sempre me incentivou e apoiou em todo meu trajeto acadêmico pela UFRGS. Obrigada Tati pela orientação e confiança.

Ao Programa de Pós Graduação em Genética e Biologia Molecular da UFRGS pela qualidade de ensino e valorização de seus estudantes.

Ao CNPq pelo apoio financeiro e licença maternidade.

À professora Dra. Lílian Eggers pelas coletas, ajuda na identificação das plantas e sugestões de grande valia para meu trabalho.

À professora Dra. Fernanda Bered pela disponibilidade e ajuda com os microssatélites.

Aos professores Dr. José Artur Chies e Dr. Giancarlo Pasquali pela disponibilidade de equipamentos.

Ao professor Dr. Thales Freitas, e as colegas Darlise e Mayara pelo auxílio com os envios de amostras para Macrogen, na Coréia.

Aos colegas de laboratório de Sistemática Molecular em especial a Eudes e ao Lauís pela ajuda em campo, no laboratório e pelas conversas e discussões durante os dois primeiros anos de doutorado. À Paula pela disponibilidade e comprometimento com a ajuda na bancada.

À Adriana Aita pelo auxílio nas coletas.

As colegas Camila, Márcia e Miriam do laboratório de Genética Vegetal pela ajuda com as análises e dicas a respeito dos microssatélites.

Aos colegas do Serviço de Imunologia do HCPA pela força nestes últimos dois anos de doutorado.

Ao Elmo pelas conversas de incentivo e apoio e pela ajuda de sempre.

A todos que contribuíram de alguma maneira para realização deste trabalho.

Muito Obrigada!

SUMÁRIO

RESUMO

Sisyrinchium palmifolium L. (Iridaceae: Iridoideae: Sisyrinchieae) é uma espécie herbácea, perene e de ampla distribuição na América do Sul. A identificação e classificação desta espécie têm sido problemáticas devido a sua grande variabilidade morfológica. Estas plantas apresentam variação tanto no hábito e porte da planta, como na morfologia foliar e no número de flores e ramificações da inflorescência. As relações filogenéticas desta espécie com outras espécies relacionadas das seções *Hydastylus* e *Viperella* também tem sido problemáticas devido à radiação recente deste grupo de plantas. Evidências filogenéticas apontam o gênero *Sisyrinchium* como monofilético e sugerem modificações na classificação de algumas seções, especialmente nos clados que incluem *S. palmifolium* e espécies relacionadas.

Com o objetivo de investigar a variabilidade genética dentro e entre as populações da subespécie típica, *S. palmifolium* subsp. *palmifolium*, presente no sul do Brasil, nove marcadores ISSR foram utilizados, em 149 indivíduos, amostrados em cinco populações (**Capítulo II**). Os resultados indicam alto grau de polimorfismo e grande variabilidade genética entre os indivíduos dentro das populações. A variação genética encontrada foi maior dentro do que entre as populações, característico de plantas de fecundação cruzada. Os dados mostram que houve uma diferenciação genética significativa ($P<0,001$) entre as populações. As análises moleculares mostraram que as populações são altamente estruturadas com baixo fluxo gênico entre elas.

Foram avaliadas as relações de parentesco entre a espécie *S. palmifolium* e espécies relacionadas das seções *Hydastylus* e *Viperella*, assim como a história evolutiva, divergência e origem dos poliploides deste grupo de plantas

(**Capítulo III**). Para isso, foram utilizados 12 marcadores filogenéticos dos genomas nuclear, plastidial e mitocondrial para um total de 44 acessos de *Sisyrinchium* da América do Sul, representando 13 espécies da seção *Hydastylus* e 11 espécies da seção *Viperella*. Nossos resultados sugerem que as duas seções correspondem a um único grupo de plantas em recente radiação adaptativa. Apesar dos principais grupos evidenciados com as análises filogenéticas serem formados por espécies da mesma seção, foi reforçada a hipótese de evolução reticulada caracterizada por eventos de alopoliploidização e introgressão. Diferentes acessos de uma mesma espécie estão dispersos em diferentes clados, exceto *S. antemeridianum,* devido a sua distribuição restrita, enquanto as espécies poliploides (*S. marchio*, *S. marchioides* e *S. weirii*), mostraram-se relacionadas às espécies diploides de ambas as seções.

Marcadores microssatélites foram isolados das espécies *Sisyrinchium palmifolium* e *S. marchioides*, provenientes do sul do Brasil (**Capítulo IV**) com o objetivo de fornecer ferramentas moleculares para investigação de relação genética entre as espécies das seções *Viperella* e *Hydastylus*. Um total de nove "primers" de SSR polimórficos foi obtido neste estudo, os quais foram testados em 24 espécies de Iridaceae, indicando que estes marcadores serão úteis em inúmeros estudos com outras espécies do mesmo gênero e outros gêneros da família tais como *Herbertia* e *Calydorea*.

Marcadores microssatélites foram utilizados para estudar os padrões de diversidade genética e fluxo gênico entre as espécies *S. palmifolium* subsp. *palmifolium*, *S. rectilineum* e *S. vaginatum* subsp. *vaginatum* no estado do Rio Grande do Sul, em 236 indivíduos, amostrados em oito populações (**Capítulo**

V). Os resultados indicam alto grau de polimorfismo e variabilidade genética entre os indivíduos dentro das populações. As populações de *S. vaginatum* subsp. *vaginatum* se mostraram mais estruturadas do que as populações de *S. palmifolium* subsp. *palmifolium*. Além disso, sugere-se que a maioria dos alelos compartilhados entre as populações é devida aos eventos de introgressão ocorridos entre estas espécies recentemente e que as relações observadas entre as populações estão de acordo com a tradicional delimitação destas espécies.

ABSTRACT

Sisyrinchium palmifolium L. (Iridaceae: Iridoideae: Sisyrinchieae) is a perennial herb with wide distribution in South America. The identification and classification of this species is problematic due to its wide morphological variability. These plants show variation in the size and habit of the plant, as in leaf morphology and inflorescence (in number of flowers and branches). The phylogenetic relationships of this species with other species of the sections *Hydastylus* and *Viperella* also has been problematic due to recent radiation of this group of plants. Phylogenetic evidences show that *Sisyrinchium* is a monophyletic genus and suggest modifications in the classification of some sections, especially in clades that include *S. palmifolium* and related species.

With the aim to investigate the genetic variability within and among populations of the typical subspecies, *S. palmifolium* subsp. *palmifolium,* from southern Brazil, nine ISSR markers were examined in 149 individuals from five populations (**Chapter II**). Results showed high degrees of polymorphism and genetic variability among individuals within populations. Genetic analyses revealed that the majority of the variation was within populations rather than among populations, that is characteristic of outcrossing plants. The data indicated significant ($P < 0.001$) genetic differentiation among populations. Molecular analysis showed that the populations are highly structured with low gene flow among them.

To evaluate the relationships among *S. palmifolium* and other species of the sections *Hydastylus* and *Viperella,* as the evolutionary history, divergence and polyploidy origin for this plant group, twelve phylogenetic markers from three genomes (nuclear, chloroplast and mitochondrial) were examined

(**Chapter III**). Forty-four accessions of *Sisyrinchium* from South America, representing 13 species of the section Hydastylus and 11 species of *Viperella*, were used in this study. Our results suggest that the two sections correspond to a single natural group of plants in recent adaptive radiation. Despite that the main groups were formed among species of the same section, it reinforced the hypothesis of reticulate evolution being characterized by introgression and allopolyploidization events. Different accessions of the same species grouped into different groups, except *S. antemeridianum* due to its restricted distribution. While, polyploid species (*S. marchio*, *S. marchioides* and *S. weirii*) showed be related to the diploid species of both sections.

Microsatellite markers were developed to *Sisyrinchium palmifolium* and *S. marchioides* from Southern Brazil (**Chapter IV**) to provide molecular tools for the investigation of the species belonging to the sections *Viperella* and *Hydastylus*. A total of nine SSR polymorphic "primers" were obtained in this study, which were tested in 24 species of Iridaceae. Cross-amplification in other Iridaceae species indicated that these markers will be useful in many studies with other species of the genus and other genera of the family such as *Herbertia* and *Calydorea*.

Microsatellite markers were used to assess the patterns of genetic diversity and gene flow among the species *S. palmifolium* subsp. *palmifolium*, *S. rectilineum* and *S. vaginatum* subsp. *vaginatum* from Rio Grande do Sul state, in 236 individuals from eight populations (**Chapter V**). Results indicated high degrees of polymorphism and genetic variability among individuals within populations. The populations of *Sisyrinchium vaginatum* subsp. *vaginatum* were more structured than *S. palmifolium* subsp. *palmifolium*. We support the

hypothesis that many shared alleles among the populations are due introgression occurred in recent past, i.e. ancestral polymorphism and that observed population relationships are congruent with traditional species delimitation.

CAPÍTULO I

INTRODUÇÃO GERAL

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1. Família Iridaceae

A família Iridaceae pertence à ordem Asparagales (APGIII, 2009) e constitui uma família relativamente grande dentre as plantas monocotiledôneas, sendo composta por sete subfamílias (Isophysiodoideae, Patersonioideae, Geosiridoideae, Aristeoideae, Nivenioideae, Crocoideae e Iridoideae), cerca de 65-75 gêneros, e aproximadamente 2000 espécies (Goldblatt *et al.*, 2008). Entre essas espécies, aproximadamente 160 ocorrem no Brasil, representando 18 gêneros, sendo 12 desses encontrados no sul do Brasil (Eggers *et al.*, 2010). Os gêneros encontrados no Brasil que possuem maior diversificação e abundância são: *Sisyrinchium* L. (58 espécies), *Neomarica* Sprague, *Pseudotrimezia* R.C. Foster (21 espécies cada um), *Trimezia* Salisb. Ex Herb. e *Cypella* Herb. (14 espécies cada um) (Souza-Chies *et al.*, 2012). As tribos Tigridieae, Sisyrinchieae e Trimezieae da subfamília Iridoideae têm representantes no sul do Brasil. Entre os mais importantes gêneros encontrados no sul do Brasil estão: *Calydorea* Ravenna (nove espécies), *Cypella* Herb. (11 espécies) e *Herbertia* Sw. (sete espécies), os quais pertencem à tribo Tigridieae, e o gênero *Sisyrinchium* (45 espécies) pertencente à tribo Sisyrinchieae (Eggers *et al.,* 2010).

Iridaceae possui ampla distribuição mundial, concentrada principalmente no Hemisfério Sul, sendo a África o maior centro de diversidade (Goldblatt, 1990) seguido pelas regiões temperadas e de altitude da América Central e do Sul. No Brasil, a família é representada por plantas nativas e exóticas, distribuídas desde a caatinga e a região do cerrado, ao longo da floresta Atlântica, e, na região Sul do país, nos campos e na floresta de Araucárias

(Eggers, 2008). Segundo Goldblatt *et al.* (2008), a divergência entre Iridaceae e seu ancestral, a família Doryanthaceae, ocorreu há aproximadamente 82 milhões de anos (no período Cretáceo) na antiga ligação entre os continentes antártico e australiano, em um período anterior a uma das grandes glaciações, o que pode vir a ser um dos motivos para uma adaptação existente em gêneros de Iridaceae que ocorrem em baixas latitudes: folhas achatadas, provavelmente para otimizar a captação de energia luminosa.

Iridaceae é constituída de plantas perenifólias ou ervas decíduas, ocasionalmente arbustos com crescimento secundário anômalo e raramente anuais (Goldblatt *et al*., 1998). As plantas são caracterizadas por apresentar, com frequência, caules subterrâneos do tipo bulbo ou rizoma e folhas cilíndricas ou planas, lisas ou plicadas. As flores apresentam perigônio de verticilos subiguais ou distintos, coloridos, androceus constituídos de três estames, livres ou unidos, e gineceu tricarpelar, trilocular, polispérmico, de placentação axial e de ovário ínfero (Eggers, 2008). As flores são dispostas em inflorescências determinadas, frequentemente muito modificadas e, às vezes reduzidas a uma flor solitária terminal. São protegidas por brácteas (escapas), sendo geralmente pediceladas. As flores são perfeitas, isto é, elas são bissexuais, com órgãos femininos e masculinos funcionais, possuindo simetria radial ou bilateral. As flores podem apresentar nectários nos septos do ovário ou nas tépalas (Goldblatt e Manning, 2008). As espécies de Iridaceae exibem uma variedade de cores e formas de flores que podem servir como sinal para os polinizadores (Goldblatt e Manning, 2008). Iridaceae é uma das poucas famílias que possuem espécies produtoras de néctar e óleo em adição as espécies que fornecem somente pólen como um recurso para o polinizador. A

família apresenta interessantes estratégias e associações com polinizadores (insetos e pássaros); um exemplo é o óleo não volátil produzido nos elaióforos (glândulas produtoras de óleo) de muitas espécies (particularmente em *Sisyrinchium*) que atraem abelhas especializadas coletoras de óleos pertencentes a duas famílias da ordem Hymenoptera, Mellitidae e Apidae (Michener, 2007).

Iridaceae possui grande interesse econômico na área da horticultura, principalmente pelo comércio de flores e pelas espécies usadas em paisagismo (por exemplo: *Gladiolus*, *Íris*, *Neomarica*, *Freesia*, *Dietes*, e outras). A família é rica em compostos fenólicos e outros metabólitos secundários que resultam em produtos utilizados na alimentação, como erva aromática e uso medicinal (como açafrão, obtidos dos estigmas de *Crocus sativus* L.), perfumaria (*Iris florentina* L.) (Dahlgren *et al.,*1985; Goldblatt e Manning, 2008).

2. Gênero *Sisyrinchium* L.

Sisyrinchium é um gênero taxonomicamente complexo da família Iridaceae pertencente à subfamília Iridoideae e tribo Sisyrinchieae, sendo restrito quase que inteiramente às Américas. A América do Sul é um centro evolutivo para Iridaceae e é, provavelmente, o lugar de origem e o centro de distribuição para o gênero *Sisyrinchium*. Inúmeras espécies desse gênero são restritas à América do Sul. Segundo Chauveau *et al.* (2011), os padrões biogeográficos observados para as espécies de *Sisyrinchium* indicam expansão do gênero na América Central e norte dos Andes até o subandes variando entre o Chile e Argentina e estendendo-se até a Bacia do Rio Paraná. (Figura 1).

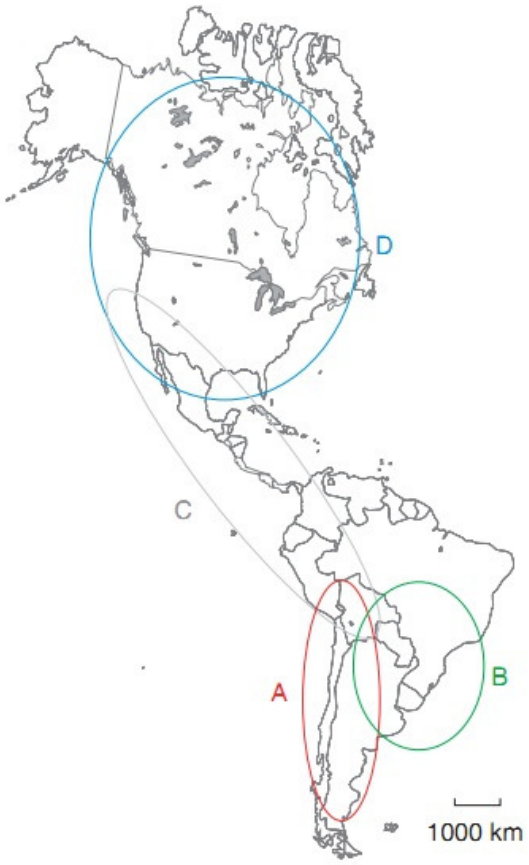

Figura. 1. Principal padrão geográfico dentro dos limites de distribuição de *Sisyrinchium*: (A) Limites de distribuição dentro do subandes, entre Argentina e Chile; (B) área de extensão até a Bacia do Rio Paraná; (C) área central de circunscrição entre o sudoeste dos Estados Unidos até a Bolívia; (D) limites de distribuição na América do Norte. (Figura retirada do artigo de Chauveau *et al*., 2011).

As espécies de *Sisyrinchium* apresentam flores amarelas, brancas, azuis claras, azuis escuras ou violetas, com tépalas subiguais, livres entre si, em disposição plana ou em taça. Os estames podem ter os filetes livres ou unidos, formando uma coluna, a qual pode ou não apresentar tricomas. Tais tricomas, conhecidos como elaióforos, são células produtoras e secretoras de óleo que surgem entre as células epiteliais (Cocucci e Vogel, 2001; Goldblatt e Manning, 2008). Estas espécies podem ser distinguidas ainda pelos traços das folhas, as quais podem ser lanceoladas a lineares ou ocasionalmente cilíndricas. O

escapo floral pode ser simples ou ramificado, frequentemente alado (Goldblatt *et al*., 1998), terminando diretamente nas sinflorescências ou em uma bráctea foliácea ereta (sinflorescência aparentemente lateral).

Sisyrinchium tem sido objeto de vários estudos, em particular ao nível citogenético, dispondo dados de quantidade de DNA e determinações de números cromossômicos (Kenton *et al*., 1986; Rudall *et al*., 1986; Corrêa, 2011; Piccoli, 2012; Souza-Chies *et al*., 2012; Tacuatiá *et al*., 2012). Tais dados que concernem em sua maioria espécies norte-americanas, mostram uma forte tendência à poliploidia e à provável origem de algumas espécies por hibridação interespecífica (Goldblatt e Takei, 1997). *Sisyrinchium* também tem sido alvo de estudos relacionados à evolução floral, tricomas produtores de óleo e elaióforos (Chauveau *et al*, 2011 e 2012; Silvério *et al*., 2012); biologia floral e polinização (Truylio *et al*., 2002); variabilidade genética (Corrêa, 2011; Tacuatiá *et al*., 2012a, 2012b e 2012c; Souza-Chies *et al*., 2012); caracterização química e morfométrica (Indrusiak, 2010).

A identificação das espécies de *Sisyrinchium* tem sido considerada extremamente complicada pelos sistematas em função da grande variação morfológica observada na natureza (Johnston, 1938; Cholewa e Henderson, 1984; Kenton *et al*., 1986; Rudall *et al*., 1986). Estudos realizados com este gênero reconhecem que o mesmo é constituído por representantes com ampla variabilidade (Souza-Chies *et al*., 2012). Alguns dos caracteres usados pelos botânicos para delimitar as espécies em *Sisyrinchium* são extremamente variáveis e geralmente de natureza quantitativa (altura da planta, por exemplo), enquanto outros são praticamente invariáveis, como a textura da superfície do pólen (Cholewa e Henderson, 1984). A gradação na expressão de um caráter

dentro de uma mesma população pode fazer com que, dependendo do tratamento taxonômico empregado, seja reconhecida a ocorrência de uma, duas ou, até mesmo, sete espécies. Segundo Henderson (1976), os caracteres taxonômicos mais seguros são encontrados nas flores e na forma da haste, podendo estes estar relacionados ao número cromossômico e sistema de cruzamento das espécies. A grande variação morfológica encontrada neste gênero parece ainda refletir uma plasticidade fenotípica de causa ambiental. Fatores como o pH do solo e estresse hídrico, possivelmente influenciam diversas características externas das plantas (Ingram, 1967; Henderson, 1976). Portanto, o número exato de espécies que compõem o gênero *Sisyrinchium* ainda não é totalmente conhecido. Conforme a literatura, esse número oscila entre 80 e 200 espécies (Rudall *et al*., 1986; Goldblatt *et al*., 2008), e a divisão infragenérica ainda não é bem definida sendo considerada insatisfatória por muitos taxonomistas (Goldblatt *et al*., 1990; Cocucci e Vogel, 2001; Ravenna, 2003). Além disso, representantes do gênero da América Central e do Sul permanecem largamente desconhecidos: 24% dos 206 táxons aceitos para a lista mundial de espécies de Iridaceae foram descritos para estas áreas durante os últimos 10 anos (Barker, 2004), sugerindo que muitas espécies ainda não foram descritas.

Ao longo dos últimos 152 anos um grande número de classificações do gênero foram propostos, baseados em diferentes tipos de características morfológicas. Klatt (1861-1862) reconheceu três seções *Sisyringium*, *Androsolen* and *Spathirhachis*, baseado na forma da folha, posição da sinflorescência e conexão dos filamentos na coluna estaminal. Baker (1877) considerou as mesmas seções reconhecidas por Klatt e criou a seção

Cephalanthum, baseado na disposição lateral da sinflorescência. Bentham e Hooker (1883) subdividiu o gênero em quatro seções: *Bermudiana*, *Echthronema*, *Eriphilema* e *Nuno*, baseado em traços florais tais como a forma, cor do perigônio e conexão dos filetes. As mais recentes classificações de Ravenna (2000, 2003) dividiram o gênero em oito seções: *Sisyrinchium*, *Echtronema*, *Spathirhachis*, *Lenitium*, *Scirpeocharis*, *Segetia*, *Hydastylus* e *Viperella*, baseado na presença ou ausência de folhas basais, inflorescência pseudolateral, características dos estames, filamentos e espata.

A filogenia de *Sisyrinchium* apresentada recentemente no trabalho de Chauveau *et al*. (2011), utilizando a combinação de oito marcadores moleculares pertencentes ao genoma nuclear, plastidial e mitocondrial, confirmou a monofilia do gênero e dividiu o gênero em nove clados. As subdivisões reconhecidas na classificação de Ravenna para o gênero (2000, 2002 e 2003) são parcialmente suportada no trabalho de Chauveau *et al*. (2011), com algumas exceções. Espécies da seção *Hydastylus* foram distribuídas entre dois clados, sendo também incluídas as espécies pertencentes à seção *Viperella* em um dos clados (Figura 2). Portanto, Chauveau *et al*. (2011) sugerem que mudanças na classificação de algumas seções são necessárias, especialmente nos clados que incluem as espécies do sul do Brasil.

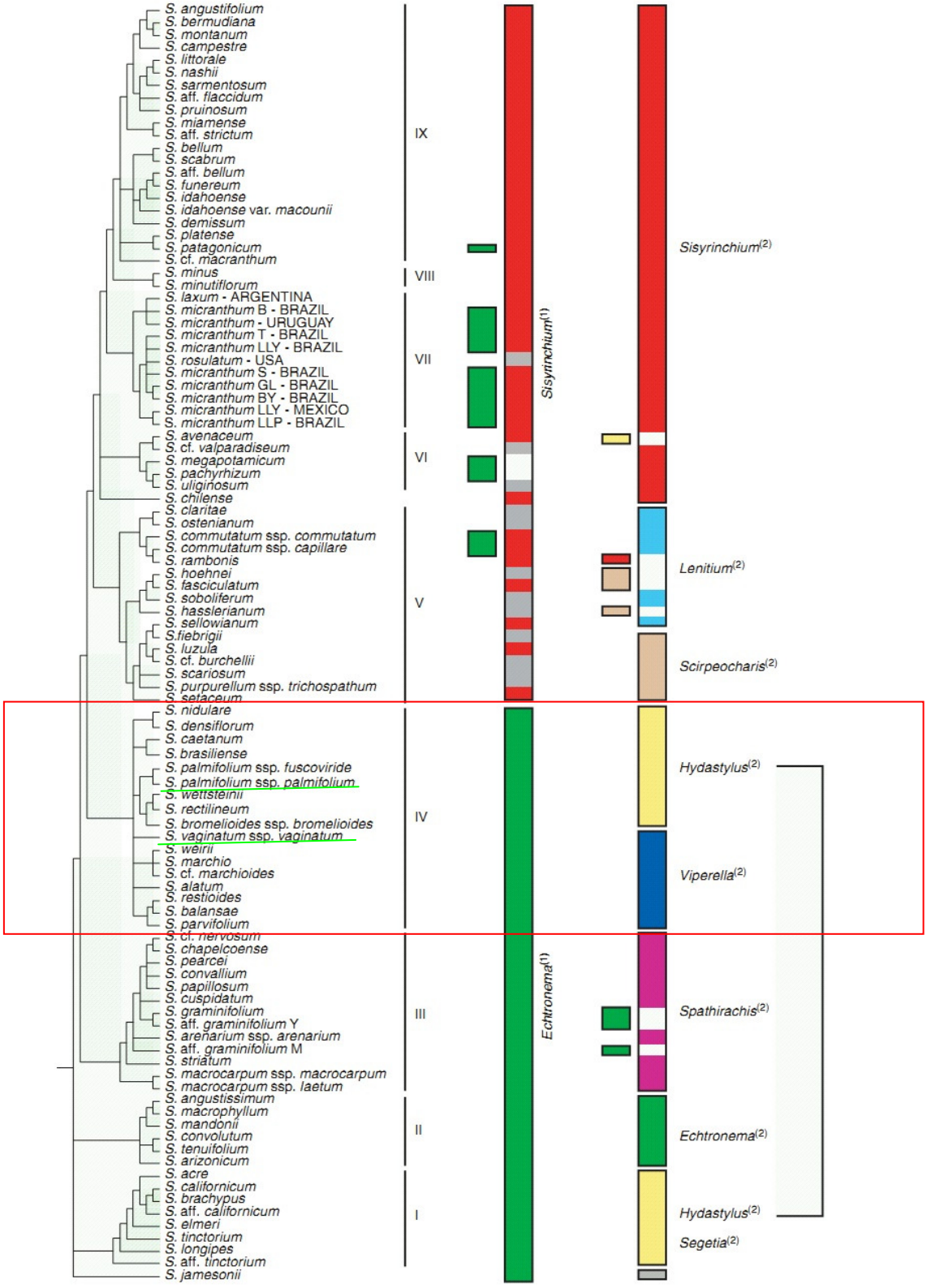

Figura 2. Posição filogenética das espécies *Sisyrinchium palmifolium* (seção *Hydastylus*) e *Sisyrinchium vaginatum* (seção *Viperella*) e espécies relacionadas que

ocorrem no sul do Brasil e Argentina, as quais formam um único clado (Clado IV) na filogenia do gênero *Sisyrinchium* realizada por Chauveau *et al*., 2011. O subgênero (1) proposto por Goldbladtt *et al*. (1990) e as seções (2) definidas como subdivisão do gênero por Ravenna (2000, 2002 e 2003), estão indicados no lado direito da figura.

3. Seções *Hydastylus* e *Viperella*

A seção *Hydastylus* se caracteriza por apresentar plantas isoladas, dispersas ou formando touceiras densas, com folhas lineares e basais. O escapo floral é longo, comprimido, distintamente alado, terminando em uma bráctea foliácea ereta. A sinflorescência é aparentemente lateral, muitas vezes congesta. As flores apresentam perigônio geralmente plano, filetes unidos por um terço ou metade do seu comprimento, sem tricomas glandulares, e ramos do estilete mais longos que a porção unida do estilete (Ravenna, 2000). O tratamento mais recente para esta seção totalizou 23 espécies, dentre as quais *S. binervatum* Ravenna, *S. brasiliense* Ravenna, *S. bromelioides* R.C. Foster, *S. caeteanum* Ravenna, *S. coalitum* Ravenna, *S. congestum* Klatt, *S. decumbens* Ravenna, *S. densiflorum* Ravenna, *S. eserrulatum* Johnston, *S. minense* Ravenna, *S. nidulare* (Hand-Mazz) Johnston, *S. palmifolium*, *S. plicatulum* Ravenna, *S. rectilineum* Ravenna e *S. wettsteinni* Dusén ocorrem no Brasil.

A seção *Viperella* caracteriza-se por plantas herbáceas, folhas basais ausentes ou muito reduzidas, apresentando caule ancipitado, com ripídio axilar ou terminal protegido por duas brácteas (Ravenna, 2003). Dentre as espécies citadas para esta seção estão: *S. vaginatum*, *S. alatum* Hook., *S. balansae* Baker, *S. marchio* (Vell.) Steud, *S. parvifolium* Baker, *S. restioides* Spreg., *S. weirii* Baker, *S. marchioides* Ravenna ocorrentes no Brasil.

As espécies destas duas seções são plantas herbáceas, perenes com flores amarelas sem elaióforos, sendo o pólen o único recurso fornecido aos polinizadores. Ambas as seções apresentam espécies no sul do Brasil com o mesmo número básico cromossômico, x=9. Estas espécies podem apresentar três níveis de ploidia: diploides (2n= 2x= 18), tetraploides (2n= 4x= 36) e hexaploide (2n= 6x= 54) (Corrêa, 2011; Picolli, 2012).

4. *Sisyrinchium palmifolium* L.

Sisyrinchium palmifolium (Figura 3) é uma espécie herbácea, perene, pertencente à subfamília Iridoideae (Iridaceae), tribo Sisyrinchieae (Goldblatt *et al.*, 1998). A espécie apresenta flores amarelas com inflorescência aparentemente lateral, o escapo floral termina em uma bráctea foliácea ereta que se assemelha a uma continuação estéril do caule, suas folhas são basais e lineares de até 80 cm de comprimento e 1-1,5cm de largura (Heaton e Mathew, 1998). *Sisyrinchium palmifolium* não apresenta glândulas ou tricomas no tubo estaminal. Sua beleza impressiona justamente, pelo porte da planta e pelo florescimento sucessivo dos botões florais. Esta espécie tem ocorrência no Uruguai, norte da Argentina, Bolívia, Peru, e no Brasil é encontrada nos estados do Rio Grande do Sul, Santa Catarina, Paraná, São Paulo, Rio de Janeiro, Minas Gerais e Bahia (Chukr e Capellari Jr., 2003). Figura 4 A. *S. palmifolium* subsp. *palmifolium* apresenta distribuição nos estados do Paraná, Santa Catarina e Rio Grande do Sul. Figura 4 B.

Sisyrinchium palmifolium, também citada como *S. macrocephalum* Graham ou como sinônimo deste (Johnston, 1938; Rambo, 1949; Teodoro Luis, 1960; Lombardo, 1984; Innes, 1985; Ravenna, 1991, 2002 e 2003; Chukr e

Capellari Jr, 2003; Eggers, 2008), apresenta-se na natureza com uma grande variabilidade morfológica principalmente no que se refere à largura, altura e textura das folhas, formato da inflorescência, forma da raiz e, apesar das flores serem sempre amarelas, eventualmente suas tépalas apresentam um arco de cor marrom. Devido a essa variabilidade, sua identificação e classificação têm sido problemáticas, o que tem permitiu a descriação de 3 diferentes subespécies, *S. palmifolium* subsp. *palmifolium*, *S. palmifolium* subsp. *fuscoviridae* Rav. e *S. palmifolium* subsp. *giganteum* Rav.

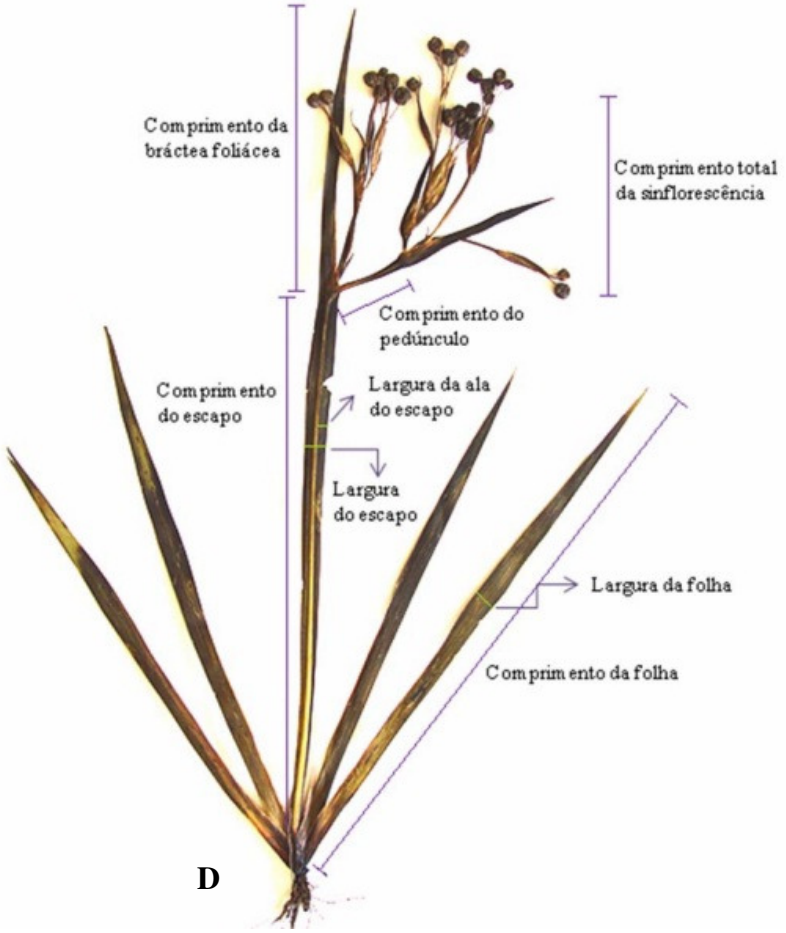

Figura 3. *Sisyrinchium palmifolium* L. (Iridaceae) **A.** hábito; **B.** inflorescência; **C.** detalhe das flores; **D.** detalhe da planta. Fotos cedidas por Lilian Eggers e Adriana Aita.

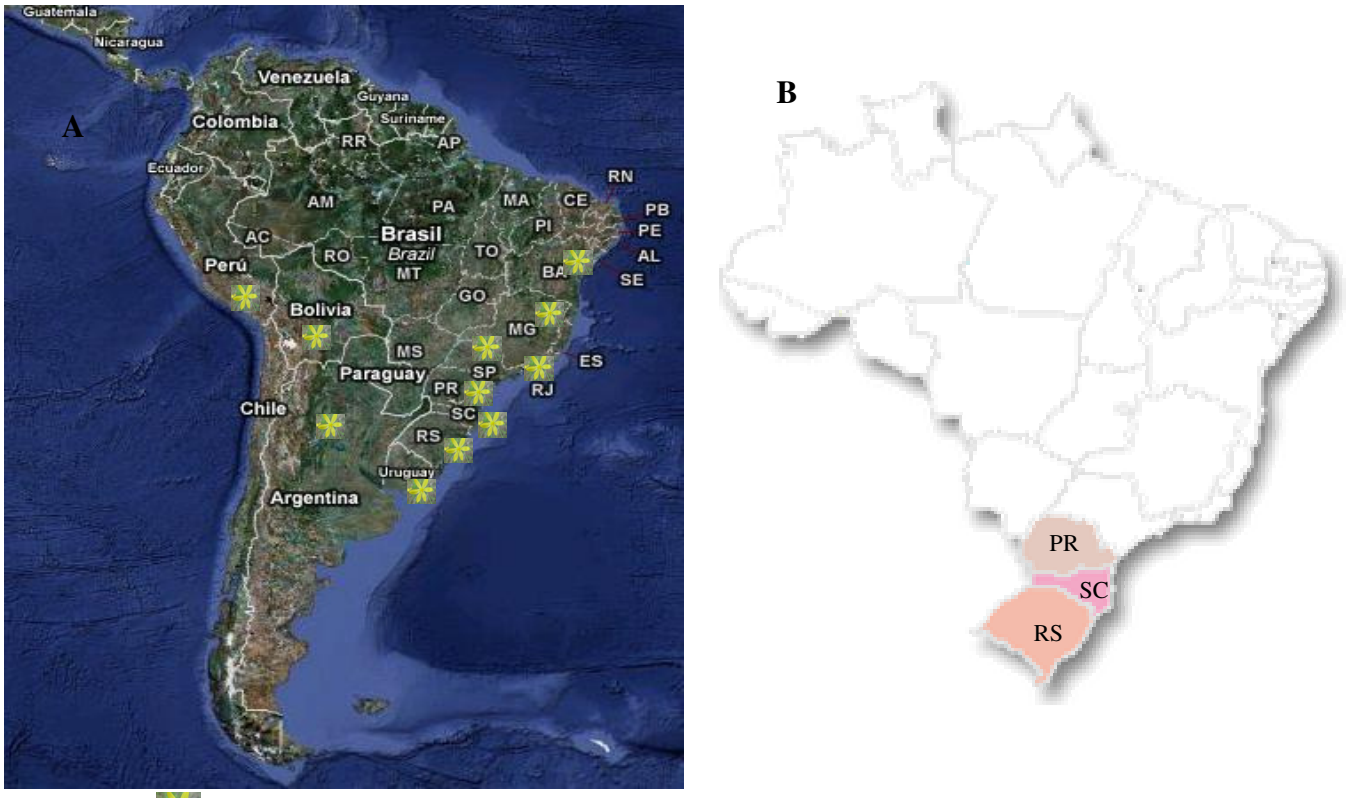

Figura 4. A. Distribuição de *Sisyrinchium palmifolium* (Chukr e Capellari Jr., 2003). B. Distribuição de *Sisyrinchium palmifolium* subsp. *palmifolium* (Eggers, 2010). (*http://floradobrasil.jbrj.gov.br/2010/FB008072*).

5. Marcadores Moleculares utilizados para Caracterização Genética de Plantas

Um grande número de marcadores moleculares, presentes nos três genomas vegetais (nuclear, plastidial e mitocondrial), tem auxiliado no esclarecimento de questões referentes à diversidade genética, estrutura populacional, filogenética e identificação de espécies. A utilização desses marcadores vem contribuindo para uma maior conservação e conhecimento da biodiversidade de diversas espécies, gêneros e famílias de plantas.

Entre esses marcadores estão os microssatélites ou SSR (simple sequence repeats) que são muito utilizados para estudos de genética de populações pelo seu alto polimorfismo, fácil genotipagem e herança codominante (Sun *et al*., 2009). Estes marcadores oferecem uma estimativa em fina escala da diversidade genética dentro e entre populações, sendo capazes de identificar padrões de fluxo gênico, rotas de dispersão e estrutura genética das espécies, sendo esses fatores essenciais para o entendimento dos processos evolutivos das espécies (Jay *et al*., 2012). Além disso, marcadores SSR podem ser utilizados para resolver problemas quanto à delimitação de gêneros e espécies, questões relacionadas à paternidade, determinação do modo de reprodução e a comparação entre espécies (McDonald e Potts, 1997; Parker *et al.,* 1998). As sequências destes marcadores são constituídas de DNA repetitivo, dispostas lado a lado, onde pequenos motivos (1 a 6 pares de base) são repetidos *n* vezes. Os microssatélites representam regiões instáveis do genoma, que estão sujeitas a alterações mutacionais, geralmente adições ou deleções de um número integral de repetições, com taxas muito mais elevadas do que as observadas em sequências de DNA não repetitivo (Jarne e Lagoda, 1996). Os SSR estão distribuídos ao longo de sequências codificantes e não codificantes do DNA (Schlötterer e Tautz, 1992). Essas regiões repetidas têm sido identificadas nos três genomas vegetais: nuclear, plastidial (Powell *et al*., 1995) e mitocondrial (Soranzo *et al*., 1999). Os marcadores SSR, especialmente aqueles desenvolvidos para espécie alvo, geralmente apresentam um grande número de alelos, incluindo alelos raros. Uma vez que o número de alelos é suscetível à deriva genética, os marcadores microssatélites são também adequados para detecção de redução da variação

genética dentro de populações que estão associadas à deriva genética (Tamaki *et al*., 2008).

Outro tipo de marcador que vem sendo utilizado para detecção de variabilidade genética de populações de plantas são os marcadores ISSR (Inter Simple Sequence Repeats). Este método utiliza um único "primer" composto por uma seqüência do microssatélite, usualmente de 16-25 pb de comprimento, sendo utilizado para amplificar principalmente as sequências Inter-SSR de diferentes tamanhos. Os alelos polimórficos ocorrem sempre que em um genoma esteja faltando a seqüência repetida ou tenha uma deleção ou uma inserção que modifica a distância entre as repetições. Embora ISSR sejam marcadores de herança dominante, têm a vantagem de analisar loci múltiplos em uma única reação (Goulão e Oliveira, 2001), possuir elevado grau de polimorfismo, reprodutibilidade e baixo custo.

Além dos marcadores SSR e ISSR, regiões codificantes e não codificantes dos três genomas vegetais têm sido utilizadas como marcadores em estudos filogenéticos, filogeográficos, na identificação de espécies (DNA *barcode*) e verificação de variabilidade genética. Entre os marcadores mais utilizados em filogenia de plantas estão os espaçadores internos transcritos ITS (internal transcribed spacers) do DNA ribossomal nuclear, que correspondem aos espaçadores intergênicos ITS1, ITS2, os quais separam os genes 18S, 5,8S e 26S. Estes marcadores possuem herança biparental e alta taxa de substituição nucleotídica sendo considerados eficientes para resolver relações filogenéticas em níveis taxonômicos mais baixos (Mort *et al*., 2007). Em relação ao genoma plastidial entre os marcadores mais utilizados em filogenia de plantas estão os espaçadores e íntrons das seguintes regiões: *rpo*C1, *rpo*B,

*mat*k, *trn*H-*psb*A, *trn*L-*trn*F plus intron *trn*L, *rpo*B-*trn*C, *trn*Q-*rps*16 and *psb*K-*psb*I (Shaw *et al*., 2005). O mtDNA de plantas é muito pouco estudado em comparação ao cpDNA. A taxa de substituição de nucleotídeos é de três a quatro vezes menor que no cpDNA (Palmer, 1992). Portanto, o mtDNA evolui mais lentamente do que os genomas do cloroplasto e nuclear e como no cpDNA, a herança materna parece predominar no mtDNA de angiospermas (Hamza, 2010).

A reconstrução filogenética tem sido considerada um dos maiores desafios da Biologia contemporânea. Entretanto, o esforço tem sido válido devido aos consideráveis benefícios que robustas árvores filogenéticas podem fornecer para diversos campos da Biologia básica e aplicada. A filogenia tem sido uma ferramenta de grande utilidade na compreensão da delimitação de espécies em vários grupos de plantas. Análises filogenéticas têm sido empregadas rotineiramente e fazem parte de estudos de sistemática na atualidade. Nas últimas décadas, a filogenia foi revolucionada pela reavaliação de suas premissas teóricas fundamentais, a começar com as publicações iniciais de Hennig (1950 e 1966), pelo uso rotineiro de dados moleculares em nível de sequência de DNA, pelo desenvolvimento de novos algoritmos eficientes, e pela disponibilidade de recursos computacionais que vem permitindo o uso de amplos conjuntos de dados.

OBJETIVO GERAL

A presente tese de doutorado está inserida em um projeto maior intitulado "Biologia e evolução das espécies brasileiras de *Sisyrinchium* (Iridaceae)". Devido à ampla complexidade taxonômica do gênero *Sisyrinchium*, grande número de espécies presentes na região sul do Brasil e a falta de informação referente à diversidade genética destas plantas, este projeto visa analisar a variabilidade genética e a estrutura populacional da espécie *Sisyrinchium palmifolium,* e sua relação com outras espécies das seções *Hydastylus* e *Viperella*, a fim de contribuir para o conhecimento da Biodiversidade, história evolutiva e de dispersão deste grupo de plantas e do gênero como um todo.

OBJETIVOS ESPECÍFICOS

- Avaliar a variabilidade intra e interpopulacional da espécie *S. palmifolium* subsp*. palmifolium* do sul do Brasil;

- Examinar os padrões de variação molecular dentro e entre as espécies das seções *Hydastylus* e *Viperella* do sul do Brasil utilizando marcadores dos três genomas (nrDNA, cpDNA e mtDNA) para esclarecer os problemas de circunscrição entre estas espécies; investigar a história evolutiva envolvendo reticulação e divergência deste grupo de plantas, e elaborar hipóteses sobre a origem dos poliploides;

- Desenvolver marcadores moleculares do tipo microssatélites para a espécie *S. palmifolium* (diploide) e *S. marchioides* (tetraploide) e testar sua transferabilidade para outras espécies de Iridaceae;

- Avaliar a variabilidade intra e interpopulacional das espécies *S. palmifolium* e *S. vaginatum* e verificar a possível existência de fluxo gênico entre estas espécies utilizando marcadores SSRs.

CAPÍTULO II

Genetic variability of *Sisyrinchium palmifolium* L. subsp. *palmifolium* (Iridaceae) in southern Brazil

A ser submetido para Botanical Journal of the Linnean Society

Genetic variability of *Sisyrinchium palmifolium* L. subsp. *palmifolium* (Iridaceae) in southern Brazil

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ABSTRACT

Sisyrinchium palmifolium is widely distributed in South America and has broad range of morphological variation. However, there have been no previous molecular or genetic population studies of this species. The present study was performed to investigate the genetic variability within and among populations of the typical subspecies, *S. palmifolium* subsp. *palmifolium*, from southern Brazil, based on inter simple sequence repeat (ISSR) markers. Five populations were sampled and a set of nine ISSR primers was selected for DNA amplification. High degrees of polymorphism and genetic variability were observed among individuals within populations. Genetic analyses revealed that the majority of the variation was within populations ($\Phi_{IS} = 0.68$) rather than among populations ($\Phi_{ST} = 0.32$) suggesting that *S. palmifolium* subsp. *palmifolium* can reproduce by outcrossing. Hierarchical AMOVA analysis indicated significant ($P < 0.001$) genetic differentiation among these populations. Molecular analysis showed that the populations are highly structured with low gene flow among them. This study contributes to the knowledge of a highly complex group of plants.

KEW WORDS: genetic diversity - gene flow - ISSR markers- mating system- population genetics.

INTRODUCTION

South America is one of the most important evolutionary centers for Iridaceae species and constitutes the center of origin and distribution for several species belonging to *Sisyrinchium* L.; within this genus a great variety of forms have developed on that continent (Cocucci & Vogel, 2001). The taxonomy of *Sisyrinchium* has been investigated based mainly on morphological traits and more recently on molecular and cytogenetic characters (Goldblatt *et al.*, 1990; Ravenna, 2002, 2003; Chauveau *et al.*, 2011; Tacuatiá *et al.*, 2012; Souza-Chies *et al.*, 2012). However, systematic knowledge of the genus remains poorly resolved partly because of the weedy, self-fertile nature and high morphological similarity among closely related taxa due to the recent adaptive radiation process undergone by these species, resulting in the emergence of species complexes (Goldblatt, 1982; Chauveau *et al.*, 2011), as in the case of *S. palmifolium* L. and its allies.

Sisyrinchium palmifolium is a perennial herb with linear-ensiform basal leaves, firm textured, up to 80 cm in length and 1–1.5 cm in width, and yellow flowers without elaiophores (oil-producing structures), so pollen is the only floral reward for pollinators, held by pseudolateral inflorescences subtended by terminal leaf-like bracts (Heaton & Mathew, 1998). *S. palmifolium* has sometimes been referred to by the name *S. macrocephalum* Graham (Ravenna, 2003; Chukr & Capellari Jr, 2003; Eggers, 2008). *S. palmifolium* has a wide distribution in South America (Heaton & Mathew, 1998), while the typical subspecies *S. palmifolium* subsp. *palmifolium* L. occurs in the Atlantic Forest and Pampas (less common) Biomes, in southern Brazil (Eggers, 2010)., *S. palmifolium* usually is found as natural populations in open areas, grasslands, wet environments, and in ruderal habitats. This species is used as an ornamental plant in gardens of local homes.

Knowledge of genetic diversity is valuable to understanding of species biological dynamics. Population genetic diversity is affected by a number of evolutionary factors, including the mating system, gene flow, geographical range, as well as natural selection (Aegisdóttir *et al.*, 2009, Byars *et al.*, 2009, Kolb & Durka, 2013). Among these factors, the geographical range of a species appears to greatly influence the level of genetic diversity within populations. Generally, widespread species tend to show more genetic polymorphism than species with a narrow distribution (Godt *et al.*, 2004, Gibson *et al.*, 2008).

Knowledge regarding the levels and spatial distribution of genetic diversity is critical in designing conservation strategies for protecting endangered species (Hamrick *et al.*, 1991). Both levels of genetic polymorphism within populations and the extent of genetic exchange among populations; i.e., population structuring across its geographical range, affect or determine the health and survival of species. Loss of genetic variation can markedly reduce both individual fitness and the ability of a population to adapt to changing environments (Frankham *et al.*, 2002).

Inter simple sequence repeat (ISSR) markers have been used for DNA population genetics studies in field crops, fruit trees, and herbs, and represent a powerful means of assessing genetic diversity and detecting similarities among and within species and even among populations and species of Iridaceae (Raycheva *et al.*, 2011, Tacuatiá *et al.*, 2012, Souza-Chies *et al.*, 2012).

The present study was performed i) to investigate the genetic variability within and among populations of *S. palmifolium* subsp. *palmifolium* from southern Brazil, using ISSR markers; ii) to infer the mating system of this species; iii) and to gain a better understanding of the genetic diversity of Iridaceae.

MATERIALS AND METHODS

POPULATION SAMPLING

Collection efforts were conducted in the states of Rio Grande do Sul (RS), Santa Catrina (SC) and Paraná (PR), Brazil, but only three accessions of *S. palmifolium* subsp. *palmifolium* were found in the states of RS, two in SC and no accessions in PR (Table 1, Figure 1). Voucher specimens were deposited in the ICN Herbarium, Instituto de Biociências, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil.

DNA ISOLATION AND ISSR-PCR AMPLIFICATION

DNA sample extraction was based on the method of Doyle & Doyle (1987) with modifications. A set of 10 ISSR primers was tested and 9 ISSR primers were selected for DNA amplification of all populations. PCR was carried out under the following conditions: 2–3 µl of DNA (30–50 ng), 2.5 µl of 10× reaction buffer, with 0.8–1 µl of dNTPs (10 mM) (Invitrogen, São Paulo, Brazil), 1.2–2.3 µl of $MgCl_2$ (50 mM), 1 µl of primer (10 pmol/µl), 0.2 µl of *Taq* DNA polymerase (5 U/µl) (CenBiot, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil), 0–1.5 µl of DMSO (4%), and the reaction mixtures were made up to a volume of 25 µl with H_2O. Amplification was performed in the Veriti® Thermal Cycler (Applied Biosystems, Foster City, CA) with the following profile: initial denaturation at 94°C for 5 min followed by 40 cycles of 94°C for 1 min, 45°C or 48°C (Table 2) for 45 s, 72°C for 2 min, and a final extension at 72°C for 5 min. PCR products were analyzed on 1.5% agarose gels and stained with GelRed (Amicon Corp., Lexington, MA). The sizes of the fragments were estimated by comparison with the 100-bp ladder (CENBIOT).

GENETIC DIVERSITY ANALYSES

As ISSR markers are dominant, each band was assumed to represent the phenotype at a single biallelic locus (Williams *et al.*, 1990). Amplified fragments were scored for the presence (1) or absence (0) of homologous bands. The resulting presence/absence data matrix of the phenotypes was analyzed and an unbiased genetic distance matrix (Nei, 1978) was generated with TFPGA (Tools for Population Genetic Analyses, version 1.3) (Miller, 1997) to construct an Unweighted Pair-group Method Arithmetic (UPGMA) average topology, which computed 1000 permutations and estimated the confidence limits of the dendrogram after allowing a 1000-replicate bootstrap test using the same program. Marker frequencies were estimated based on the Lynch & Milligan (1994) Taylor expansion estimate.

The similarity matrix was based on Jaccard coefficients and the dendrogram was created with the UPGMA method. A triangular matrix was computed from the clustering matrix to assess the goodness of fit between the matrix of genetic similarity and the dendrogram. All of these analyses were performed using NTSYS-PC (Numerical Taxonomy and Multivariate Analysis System for Personal Computers) software, version 2.1 package (Rohlf, 2000).

GeneAlex 6.41 was used to compute the F-statistics, providing the Fst *estimator* Φ_{ST} for dominant markers (Hartl & Clark, 1989). The Φ_{ST} values are analogous to traditional F-statistics, such that increasing positive Φ_{ST} value (between 0 and 1) indicates increasing genetic differentiation among populations. Analysis of Molecular Variance (AMOVA) was conducted using GeneAlex 6.41 based on the pairwise squared Euclidean distances among molecular phenotypes. The AMOVA package was used to calculate variance components and their significance levels (based on permutation

procedures) for the hierarchical levels, such as among populations and among individuals within populations.

To test the correlation between genetic and geographic distances in km among populations, the Mantel Test was performed using GeneAlex 6.41 (Peakall & Smouse, 2006) with 10000 permutations.

Assuming Hardy–Weinberg equilibrium, POPGENE Version 1.32 (Yeh *et al.*, 1999) was used to calculate various genetic diversity parameters: (1) the percentage of polymorphic loci; (2) Nei's gene diversity (*h*), (3) Shannon's information index (*I*) (Lewontin, 1972), gene differentiation coefficient (G_{ST}), and gene flow (Nm) (Weir & Cockerham, 1984).

The BOTTLENECK software (Luikart & Cornuet, 1999) was used to determine whether populations had recently passed through a bottleneck. Both the stepwise mutation model (SMM) and the infinite allele model (IAM) were run to calculate the heterozygosity (Heq) expected at mutation–drift equilibrium. To be statistically conservative, one should only use the SMM when analyzing microsatellite data, but because the true model of mutation for most loci is intermediate between IAM and SMM, the use of both models is recommended (Luikart & Cornuet, 1998). ISSR and microsatellites are of similar origin (i.e., simple sequence repeats) (Godwin *et al.*, 1997) and should follow the same mutation model.

STRUCTURE version 2.3.3 (Hubisz *et al.*, 2009) was employed to obtain additional insights regarding gene flow and population subdivision. The most likely number of populations (*k*) was estimated under the admixture model and correlated allelic frequencies with no prior information on population origin. To determine the most likely number of clusters (*k*), our data were conditioned on different values of *k* ranging from 1 to 8. Analyses were carried out under the admixture model assuming

independent allele frequencies and using a burn-in period of 1000, run length of 10000, and 10 iterations per *k* to confirm stabilization of summary statistics (Pritchard *et al.*, 2000). To determine the most likely number of clusters (*k*), the results generated by STRUCTURE were subsequently analyzed with STRUCTURE HARVESTER version 0.6.92 (Earl & vonHoldt, 2012) according to the method of Evanno *et al.* (2005), which is based on an ad hoc measure of Δk that evaluates the second-order rate of change of the likelihood function with respect to *k*.

RESULTS

ISSR MARKERS

Analysis of the nine ISSR primers detected 183 computable bands, of which 99.45% were polymorphic. The ISSR fragments generated an average of 20.3 bands per primer, varying from 11 to 42. The size of the amplified products ranged from 280 to > 2080 bp (Table 2).

The UPGMA dendrogram generated by NTSYS showed clusters of individuals corresponding to their respective populations (Figure 2). The dendrogram produced by TFPGA (not shown) based on Nei's unbiased genetic distance matrix presented one main clusters, comprising the populations of Rio Grande do Sul (RS1, RS2 and RS3) (bootstrap value of 78%).

The Mantel test showed no significant correlation between geographic and genetic distances (r = 0.3, P = 0.264), suggesting that the contemporary population structure is inconsistent with the isolation by distance model (Ellstrand & Elam, 1993).

POPULATION GENETIC DIVERSITY AND POPULATION STRUCTURE

High degrees of polymorphism and genetic variability were detected among individuals within populations (Table 3). Among the five populations examined, Aceguá showed the highest level of genetic variation followed by the populations collected in Porto Alegre, whereas the populations of Santa Catarina showed the lowest indices of genetic diversity (Table 3).

AMOVA generated Φ statistics (Table 4) analogous to Wright's F-statistics. The analysis revealed that approximately 32% (Φ_{ST} = 0.32) of the genetic diversity could be attributed to divergence among populations, whereas 68% was estimated to be within

population (Φ_{IS} = 0.68). The data indicated well-structured sites and significant genetic differentiation among these populations ($P < 0.001$).

S. palmifolium subsp. *palmifolium* populations showed a heterozygosity deficiency ratio that significantly deviated from the expected ratio (1:1) at mutation–drift equilibrium when either the SMM or the IAM was assumed ($P < 0.05$), suggesting that a historical expansion may have occurred in the populations (Table S1 – Supplementary data).

The k = 5 model was the most adequate for elucidating clustering. The decision was made based on the k statistic, in that the uppermost peak of its modal value corresponded to the number of clusters detected by STRUCTURE (Figure 3). Clustering into five groups corresponded exactly to the UPGMA produced by NTSYS (Figure 2). Molecular analysis showed that the populations are highly structured with low gene flow among them (Nm= 0.9488).

DISCUSSION

ISSR markers revealed a high level of genetic variation in natural populations of *S. palmifolium* subsp. *palmifolium*, with 99.45% of bands displaying polymorphism. Our analyses based on different statistics indicated that the majority of the variation was within rather than among populations. The data showed well-structured sites (Φ_{ST} = 0.32) and significant genetic differentiation between these populations ($P < 0.001$). A similar genetic population structure, determined using ISSR markers, was found in previous studies on Iridaceae species of southern Brazil performed by our team (*Sisyrinchium micranthum* Cav., *Sisyrinchium sellowianum* Klatt, and *Sisyrinchium vaginatum* Spreng.) as well as genera *Calydorea* Herb. and *Cypella* Herb.) (Souza-Chies *et al.*, 2012). Tacuatiá *et al.* (2012) analyzed five populations of *S. micranthum* with six

ISSR markers and showed a high level of polymorphism (98.75%) and highly structured sites (Φ_{ST} = 0.3372, $P < 0.001$) corresponding to different populations. Variance within populations, besides accounting for about 65–66% of the total variation, corresponded well to the genetic structure of outcrossing plants as verified in the present study for *S. palmifolium*. Outcrossing may be promoted mainly by different pollinators in *Sisyrinchium* species; e.g., *S. micranthum*, oil-collecting bees of the tribe Tapinotaspinidi (Apidae), a group predominantly distributed in southern South America (Cocucci & Vogel, 2001; Truylio *et al.*, 2002), syrphids, and small pollen-collecting bees (Truylio *et al.*, 2002; Freitas & Sazima, 2006); *S. palmifolium*, pollen-collecting polylectic bees (Cocucci & Vogel, 2001); and *S. vaginatum*, small pollen-collecting bees (Barbosa, 1997) and syrphids (Freitas & Sazima, 2003).

In general, long-lived perennial species, such as *S. palmifolium* and *S. vaginatum*, usually present higher levels of intraspecific genetic diversity (Nybom, 2004), which can be explained by outcrossing behavior, long-lived individuals, and overlapping generations.

Genetic differentiation among *S. palmifolium* subsp. *palmifolium* populations showed that the populations are highly structured with low gene flow among them, what also was found to *S. micranthum* in study realized by Tacuatiá *et al.*, 2012.

Widespread species, such as *S. palmifolium*, are expected to have effective long-distance dispersal mechanisms to retain their species unity through gene flow (Takayama *et al.*, 2008). The populations of many species of angiosperms are spatially isolated, often by several hundred of meters or more. As seed dispersal represents the only way in which populations can exchange individuals or colonize empty but suitable habitats (Cain *et al.*, 2000), seed dispersal ability has a particularly strong influence on

regional distribution (Murray, 1986). The seeds of most plants of all growth forms can travel only limited distances (Howe & Smallwood, 1982; Willson, 1993; Cain *et al.*, 1998). The low levels of gene flow among populations observed here can be attributed to pollen movement and pollinator behavior, because collection sites of *S. palmifolium* subsp. *palmifolium* were in areas of agriculture, grasslands, and hills, which reduced population size and probably gene flow among populations. In addition, the possibilities of pollen and seed flow among populations are expected to be low, due to the large distances among the sampled populations; e.g., Aceguá and Porto Alegre populations were separated by 325 km; Porto Alegre and Campo Alegre by 460 km; and Aceguá and Campo Alegre by 763 km. The two Porto Alegre populations were only 12 km apart, but were collected in a hilly region, which would make gene flow difficult. According to Vogel (1978), there is a decline in the diversity of polylectic bees at high altitudes; this may be responsible for the low variability seen in these areas.

The analyses indicated no significant correlation between geographic and genetic distances for the four populations, as indicated by the higher phenetic relationship between Aceguá and Morro do Osso – Porto Alegre populations, than between Morro do Osso and Morro Santana populations of Porto Alegre. The lack of correlation may be due to several evolutionary processes, such as a habitat fragmentation.

Successful pollinator-mediated fertilization depends not only on pollinators but also on the plant mating system and the origin of the pollen. Mating systems and the rate of fruit set are also often correlated with plant life forms and other life history traits (Sutherland *et al.*, 1999). Insect-pollinated perennial plants, such as *S. palmifolium*, are predicted to have self-incompatibility mechanisms to reduce the risk of genetic load

(Morgan *et al.*, 1997). However, plants often reproduce through mixed mating, being the most commonly cited mechanism that promotes stable mixed mating systems: inbreeding depression, biparental inbreeding, polygenic control of the selfing rate, fluctuating pollinator availability, and context-dependent selection against selfed offspring (Busch *et al.*, 2010). Given the many potential agents of selection in these models, mixed mating is often viewed as an evolutionary stable state resulting from selection for traits that maximize outcrossing when pollination conditions are favorable, but ensure reproduction when outcross pollinations opportunities are limited. Here, we hypothesized that the two accessions ESC 320 (SC1) and ESC 650 (ESC2) possess high degrees of polymorphic differentiation (Table 3) due to human activity, such as construction of a route that would have divided the population or intentional cultivation in urban areas, both situations that could result in limited pollination opportunities leading to a recent breakdown of self-incompatibility resulting in a transitional stage during the evolution of higher selfing rates.

CONCLUSIONS

Our results indicated that ISSR are sufficiently informative and powerful to assess genetic variability, gene flow, and suggest outcrossing as breeding system in natural populations of *S. palmifolium* subsp. *palmifolium.* This plant species exhibits a high degree of genetic variability within populations, which is very important for adaptation to the constant climate changes and for long-term survival of this species. Genetic differentiation among *S. palmifolium subsp. palmifolium* populations showed that the populations are highly structured with low gene flow among them. The findings of the present study will contribute to a better understanding of this species and may facilitate future investigation of taxonomic, due the power fingerprint of ISSR marker, phylogenetic, and ecological aspects of the species and the genus.

ACKNOWLEDGMENTS

We thank the researchers Adriana Aita, Lauís Brisolara-Corrêa and Eudes Alves who kindly contributed with assistance in field trips and for their valuable help. We also thank the CNPq, CAPES/COFECUB, and FAPERGS for financial support.

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TABLES AND FIGURES

TABLES

Table 1. Populations of *Sisyrinchium palmifolium* subsp. *palmifolium* collected in southern Brazil.

Population Identification	Sample site	Population (Voucher)	Latitude (°S)	Longitude (°W)	Number of individuals
RS1	Aceguá, RS	ESC 469	31° 39' 00.8"S	54° 09' 09.1"W	30
RS2	Porto Alegre, RS Morro do Osso	AITA 19	30° 07'23.47"S	51°13'36.73"W	30
RS3	Porto Alegre, RS Morro Santana	ESC 586	30º 03' 35.2"S	51º 07' 28.1"W	31
SC1	Campo Alegre, SC	ESC 320	26° 10' 13.9"S	49° 13' 59.6"W	28
SC2	Campo Alegre, SC	ESC 650	26° 10' 16.5"S	49° 14' 04.3"W	30
	Total				149

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

Primer	Sequence (5'-3')	Annealing temperature (°C)	Number of markers	Size range (bp)
F3	(AG)8C	48	14	400-2000
F4	(GA)8C	48	16	300 - >2080
F11	(GACA)4	48	15	700 - >2080
P1	(AC)6	48	14	500- >2080
SM1	TC(AC)7A	48	15	400- >2080
SV3	(AC)8A	48	11	600-2000
SV4	A(TG)7G	48	34	470- >2080
SV5	(TG)8A	45	22	400- 800
SV8	(GA)8AC	48	42	280- >2080
Total			183	

Table 3. Indices of genetic diversity for populations of *Sisyrinchium palmifolium* subsp. *palmifolium.*

Identification Population	Percentage of polymorphic loci (%)	Nei's diversity (*h*)	Shannon index (*I*)
RS1	58.47	0.1399	0.2238
RS2	53.01	0.1382	0.2175
RS3	50.82	0.1399	0.2183
SC1	44.21	0.1293	0.1984
SC2	18.58	0.0498	0.0776

Table 4. Analyses of molecular variance (AMOVA) of *Sisyrinchium palmifolium* subsp. *palmifolium*.

Source of Variation	df	Sum Squares	Φ Statistcs	*P*
Among populations	3	901.168	Φ ST =0.32	<0.001
Within populations	145	2460.436	Φ IS =0.68	
Total	148	3361,604		

FIGURES

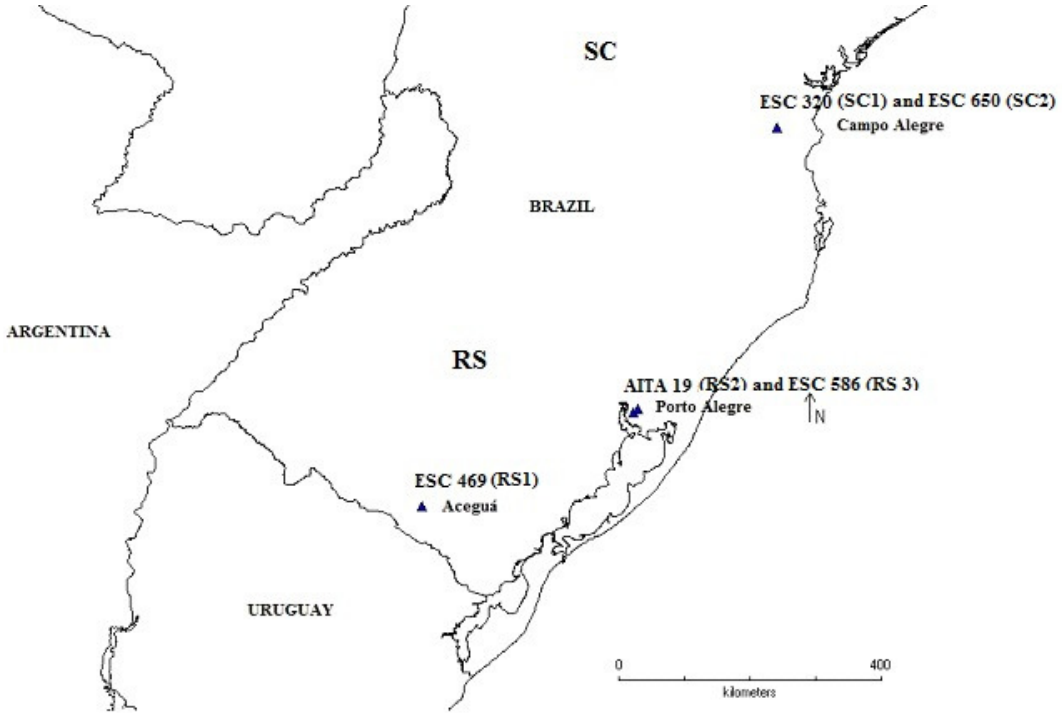

Figure 1: Map of southern Brazil, showing the sites of collection of the populations studied.

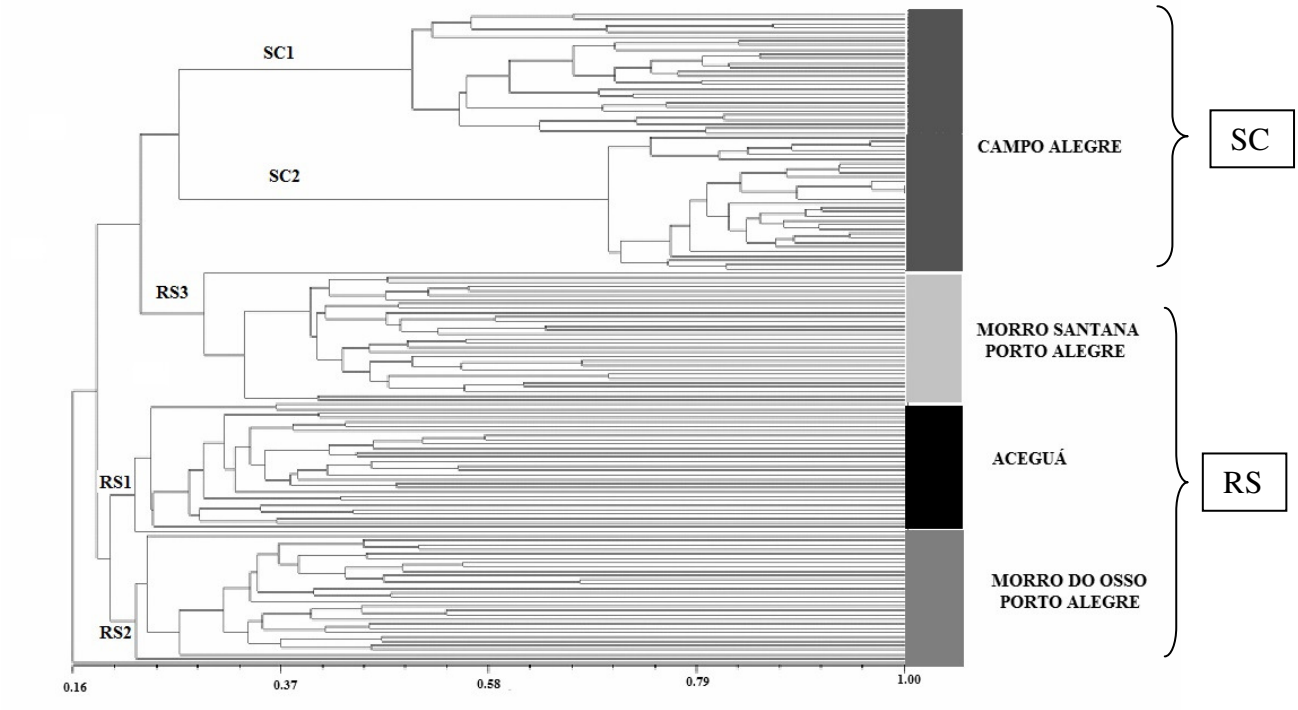

Figure 2: Dendogram generated for 149 individuals of *Sisyrinchium palmifolium* subsp. *palmifolium* of five populations. A scale of genetic distance is provided at the base of the dendrogram.

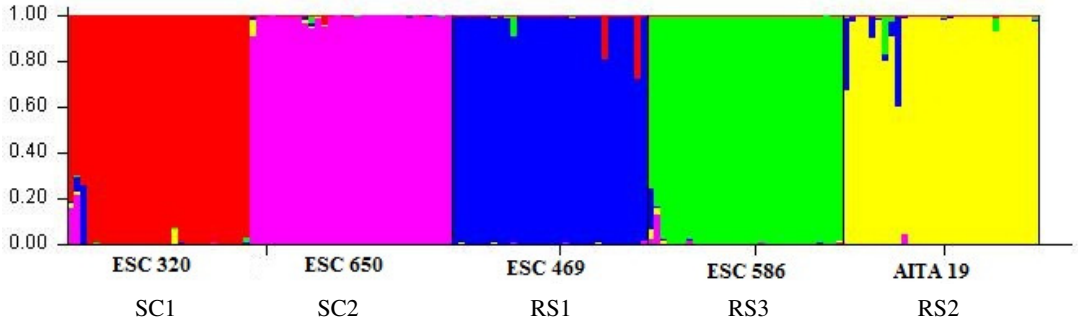

Figure 3: Bayesian admixture proportions (Q) of individual plants of *Sisyrinchium palmifolium* for $k = 5$. Each individual is represented by a single vertical line broken into k colored segments, with lengths proportional to each of the k-inferred clusters. The most likely number of populations (k) was estimated with the admixture model and correlated allele frequencies, with no prior information regarding population origin.

SUPPLEMENTARY DATA

Table S1. Results of bottleneck analysis of five accessions of *Sisyrinchium palmifolium* subsp. *palmifolium* from southern Brazil. Deviations from the mutational equilibrium, $P < 0.05$.

POPULATION	**I.A.M.**				**S.M.M.**			
	Exc. *H* exp	**Def. *H* obs**	**Exc. *H* obs**	***P***	**Exc. *H* exp**	**Def. *H* obs**	**Exc. *H* obs**	***P***
1. Sign test								
ESC 320	84.81	138	45	0.00000	95.03	141	42	0.00000
ESC 650	84.97	138	45	0.00000	95.31	141	42	0.00000
ESC 469	78.24	129	54	0.00015	91.78	136	47	0.00000
ESC 586	78.07	131	52	0.00005	90.92	139	44	0.00000
Aita 19	78.57	130	53	0.00007	91.51	139	44	0.00000
2. Standardized differences test (T2 values)								
ESC 320	-10.230			0.00000	-12.268			0.00000
ESC 650	-10.206			0.00000	-13.868			0.00000
ESC 469	-8.458			0.00000	-12.024			0.00000
ESC 586	-8.458			0.00000	-12.001			0.00000
Aita 19	-8.892			0.00000	-12.483			0.00000
3. Wilcoxon test (probabilities – two tail for *H* excess or deficiency)								
ESC 320				0.00000				0.00000
ESC 650				0.00000				0.00000
ESC 469				0.00000				0.00000
ESC 586				0.00000				0.00000
Aita 19				0.00000				0.00000

CAPÍTULO III

Tracing the nucleotide diversity and relationships inferred for two sections of *Sisyrinchium* (Iridaceae) from Southern Brazil

A ser submetido para Plant Systematics and Evolution

Tracing the nucleotide diversity and relationships inferred for two sections of *Sisyrinchium* (Iridaceae) from Southern Brazil

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Abstract

Sisyrinchium L. (Iridaceae) is a monophyletic genus consisting about 200 species, been the most important genus in terms of diversification and abundance in Brazil. Their evolutionary history has been impacted by polyploidy, hybridization and recent adaptive radiation, events which have become painful to reconstruct the phylogeny of several groups of plants. Tree topology in recent phylogenetic studies of the genus showed low resolution for the species previously classified in the sections *Hydastylus* and *Viperella*. Our study aims to make hypotheses about relationships among species, the complex evolutionary history and polyploidy origins for this group of species. The results strongly suggest that the two sections do not correspond to two clades but only a single natural group of plants in recent adaptive radiation. However, the main groups were observed among species belonging to the same section. We reinforced the hypothesis of

past reticulation events (introgression) in this taxonomic group evidenced by intraspecific variation, with different accessions of the same species grouped into different clades, except for *S. antemeridianum* an endemic species of the Highlands of Southern Brazil. We suggest that the polyploid species (*S. marchioides*, *S. marchio* and *S. weirii*) were independently derived from allopolyploidization events followed by divergent speciation.

Kew words: Allopolyploid - Evolutionary relationship - Introgression - Section *Hydastylus* - Section *Viperella* - Molecular markers

1. Introduction

The usual assumption that species have evolved from a common ancestor by a simple branching process where each branch is genetically isolated has been challenged by the observation of frequent hybridization between species in natural populations (Xu 2000). The significant and extent natural hybridization in angiosperm evolution has been widely recognized (Paun et al. 2007; Wissemann 2007), with an estimated of 25% in vascular plants forming hybrids with other species (Mallet 2005) and perhaps 11% of plant species having arisen as a result of hybridization (Ellstrand et al. 1996). In addition to hybridization, gene flow between species via introgression is a common event, with the genomes of many species apparently permeable to alleles from related species (Baack and Rieseberg 2007; Lexer et al. 2009). The phenomena of hybridization and introgression can confound efforts to reconstruct the phylogeny of such groups. According to Mason-Gamer et al. (2010) the untangling reticulate relationships among species presents an interesting challenge to systematists, and an opportunity to uncover previously undetected evolutionary processes. Comparisons among gene trees can clarify historical relationships among species, and the examination of topological conflicts among trees can reveal complicating factors such as retention of ancestral genetic polymorphism, past or ongoing gene exchange, allopolyploidy, or a combination of these factors. Distinguishing among potential causes of phylogenetic conflict is often difficult, but careful comparisons among trees can help the identification of the involved species, and allow specific hypotheses to be formulated. Many inferences of reticulate evolution have been based on comparisons among gene trees; in plants, comparisons between chloroplast DNA (cpDNA) and internal transcribed spacer (ITS) of the ribosomal nuclear DNA phylogenies are especially widely used and some works have also including information from the mitochondrial

genome (Chat et al. 2004; Mansion et al. 2005; Guggisberg et al. 2006; Govindarajulu et al. 2011; Willyard et al. 2011). Moreover, adaptive radiations are a major challenge to molecular systematics because individual gene lineages may be so recent that they fail to coalesce before the time of species divergence (Edwards et al. 2007). Unfortunately, as taxa become more closely related, their phylogenetic histories become more difficult to reconstruct because of the lack of variation necessary to trace their evolutionary relationships. Among recently diverged species, genealogies inferred from independent genomic regions are likely to disagree due to the differential sorting of ancestral polymorphism into daughter lineages such that each gene tree may differ from the species tree (Goodman et al. 1979; Maddison 1997; Funk and Omland 2003; Degnan and Rosenberg 2006). Because lineage sorting may be much more prevalent when speciation is rapid, multiple independent loci will likely provide the most robust data for phylogenetic reconstruction of lineages that have undergone rapid speciation (Schaal and Olsen 2000; Beltran et al. 2002; Cronn et al. 2002; Shedlock et al. 2004; Barker et al. 2005). Multiple accessions per species can help to infer evolutionary relationships of recently diverged species (Edwards and Beerli 2000; Maddison and Knowles 2006).

The genus *Sisyrinchium* L. (Iridaceae: Iridoideae: Sisyrinchieae) currently consists of about 200 species (Goldblatt et al. 2008), been the most important Iridaceae genus in terms of diversification and abundance in Brazil (Souza-Chies et al. 2012). *Sisyrinchium* has been suggested to be of South American origin (Goldblatt et al. 2008; Chauveau et al. 2011). For the Southern Hemisphere, its biogeographical pattern indicates expansions from Central America and the northern Andes to the subAndean ranges between Chile and Argentina and to the area of the Paraná river basin (Chauveau et al. 2011). The evolutionary history of *Sisyrinchium* has been impacted by polyploidy, hybridization, divergent allopatric species diversification and recent adaptive radiation

process (Chauveau et al. 2011; Souza-Chies et al. 2012; Tacuatiá et al. 2012a and 2012b), suggesting that this genus is an ideal group to investigate polyploidy origin and the complexities of reticulation events.

Phylogenetic study of *Sysirinchium* realized recently combined eight molecular markers of the plastidial, mitochondrial and nuclear genomes for their analysis of data (Chauveau et al. 2011). This study confirmed the monophyly of the genus and retrieved nine major clades weakly connected to the subdivisions previously recognized. The most recent classification of Ravenna (2000 and 2003) that divided the genus into eight sections was partly supported by the study of Chauveau et al. (2011), with some exceptions. Chauveau et al. (2011) suggested that changes in the classification of the sections are needed, especially in the clades including species from southern Brazil. Species of section *Hydastylus* were distributed among two clades being one formed by North American species in majority, and the other formed by species of Southern Brazil and one species of Argentina, the latter also includes the species forming section *Viperella*, all from Southern Brazil. This was unanticipated, according to Chauveau et al. (2011), since the latter section is characterized by distinctive morphological characters, such as absent or highly reduced basal leaves, and caulescent plants bearing many short caulinate leaves (Ravenna 2003). According to Chauveau et al. (2011), this latter clade is a well-supported monophyletic group, its status as a section should be considered.

Species related to *Sysirinchium palmifolium* L. (section *Hydastylus*) and *S. vaginatum* Spreng. (section *Viperella*) are perennial herb with yellow flowers without elaiophores (oil-producing structures), been the pollen the only floral reward to pollinators. Both groups of species have high morphological similarity among closely related taxa within their sections. Tree topology in recent phylogenetic studies

(Chauveau et al. 2011) showed low resolution in the clade that included the sections *Hydastylus* and *Viperella*, which can be attributable to a recent adaptive radiation of *Sisyrinchium* genus (Goldblatt 1982). Furthermore, both sections showed the same basic chromosome number, x=9, occurring three ploidy levels: diploid (2n= 2x= 18) to all species related to *S. palmifolium* and species related to *S. vaginatum*, tetraploid (2n= 4x= 36) in *S. marchio* (Vell.) Steud. and *S. marchioides* Ravenna, and hexaploid (2n= 6x= 54) in *S. weirii* (Corrêa 2011; Picolli 2012).

Given the limitations of morphological characters to circumscribe species belonging to the sections *Hydastylus* and *Viperella*, recent adaptive radiation and low phylogenetic resolution (Chauveau et al. 2011; Souza-Chies et al. 2012), we increased the number of species and accessions by species and added new molecular data to elucidate the backbone phylogeny as well as to ascertain phylogenetic relationships within and among species to provide solid foundations for species delimitation.

The current study employs molecular data to examine patterns of variation within and among species of sections *Hydastylus* and *Viperella* from Southern Brazil. The specific goals were to (1) determine which markers (nrDNA ITS, cpDNA regions and mtDNA regions) show the most number of polymorphic characters that can help to elucidate the circumscriptions problems concerning the species of sections *Hydastylus* and *Viperella*, (2) investigate the complex evolutionary history involving reticulation and divergence in this group of plants and (3) make hypotheses about polyploidy origins.

2. Materials and Methods

2.1 Taxon Sampling and Molecular Tools

A total of 44 accessions of *Sisyrinchium* from South America, representing 13 species of the section *Hydastylus* and 11 species of section *Viperella* were sampled. In

addition, *S. fiebrigii* I.M.Johnst. and *Sisyrinchium sp.* were included as outgroup. Taxa sampled, voucher information and GenBank accession numbers are listed in Table 1. The distinct data sets were treated with a different number of taxa (Table 1).

Total genomic DNA was extracted from fresh or silica gel-dried leaves using the CTAB method (Doyle and Doyle 1987).

Twelve markers were used in this study being four additional markers used for the first time to this group of species (indicated with an asterisk). Three coding plastid DNA regions (*rpo*C1, *rpo*B and *mat*k), five plastid DNA intergenic spacers and one intron (*trn*H-*psb*A, *trn*L-*trn*F* plus intron *trn*L*, *rpo*B-*trn*C*, *trn*Q-*rps*16 and *psb*K-*psb*I*), three mitochondrial DNA introns (*nad*1 - 2/3, *nad*4 - 1/2 and *nad*5 - d/e*) and the nuclear ribosomal DNA internal transcribed spacer (ITS) regions, including ITS1, ITS2 spacers and 5.8S gene, were used in this study. Some DNA sequences used by Chauveau et al. 2011 were obtained of the GenBank as indicated in Table 1. Primers used to amplify each DNA region and additional primers used for sequencing are given in Table S1 in the Supplementary Data.

PCR amplifications were perfomed using a Vereti thermal cycler in 25 µL total volume reaction with the following reactions components: 1 µL of genomic DNA (approx. 30-50ng), 2.5 µL of reaction buffer 10x, 0.75 µL of $MgCl_2$ (50mM), 0.5 µL of each primer (10 pmol/µL), 0.5 µL of dNTP (10mM), 0.25 µL of Taq DNA polymerase (5U/µL) (CenBiot, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil) and 2.5/1.0 µL of DMSO (96%). The detailed PCR conditions for each DNA locus used in this study are given in Table S2 in the Supplementary Data. The PCR amplified products were purified with shrimp alkaline phosphatase and exonuclease I (Amersham Biosciences). DNA sequencing was performed bidirectionally in an ABI 3130XL (Applied Biosystems).

2.2 Sequence alignments and polymorphism markers

Sequences were analyzed and edited by Chromas version 1.45 software (McCarthy 1996 -1998). The sequence alignments were done using the Clustal X 2.0 program (Larkin et al. 2007) and manually refined with the help of GeneDoc (Nicholas and Nicholas 1997) and BioEdit softwares (Hall 1999).

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 plants belonging to sections *Viperella* and *Hydastylus* based on Ravenna's classification.

2.3 Phylogenetic analyses

All twelve DNA regions were first analyzed independently (results not shown) and further combined in five partition data: ITS (ITS1, 5.8S, ITS2), cpDNA spacers and introns (COMB1), mtDNA regions (COMB2), *trn*L-*trn*F plus intron *trn*L, ITS, *trn*H-*psb*A, *psb*K-*psb*I, *rpo*B-*trn*C plus *nad*5 (COMB3), and all twelve DNA regions (COMB4).

The Maximum parsimony (MP) analyses were conducted in PAUP 4.0b10 version (Swofford 2002). All the MP analyses used heuristic searches with 1000 random addition replicates, tree bisection reconnection (TBR) branch swapping. The indels of the COMB3 and COMB4 data were treated as "missing" data, while the indels of ITS, COMB1 and COMB2 data were coded as binary characters once the indels showed a higher resolution in the trees. Majority rule consensus tree were calculated from all most-parsimonious trees. The robustness of nodes was evaluated with 1000 bootstrap replicates of new heuristic searches (100 random addition replicates, TBR

branch swapping, multrees off). The relative proportion of homoplasy in the MP analyses was estimated through Consistency and Retention Indexes (respectively CI and RI) available in PAUP tools.

Maximum likelihood (ML) (Felsenstein 1981) and Bayesian Inference (Yang and Rannala 1997) analyses were performed. Substitution models for each data set or data partition (Table 2) used in the analyses was obtained using AIC criterion in MrModeltest 2.3 (Nylander 2004). The selected models were: GTR + I + G (ITS and COMB1), F81 + I (COMB2) and HKY+ I + G (COMB3 and COMB4).

ML analyses were performed using PhyML 3.0 online web server (Guindon et al. 2005) on data matrices excluding the coded indels, since indels cannot be dealt with in PhyML. The reliability of ML topologies was assessed by bootstrap tests using 1000 replicates. Bayesian inference (BI) was performed using MrBayes 3.0b4 for Windows (Huelsenbeck and Ronquist 2002) to generate a posterior probability distribution using Markov Chain Monte Carlo (MCMC) methods, with the evaluation of at least 10^6 generations and its critical value was set to stop the analysis automatically when the standard deviation of split frequencies had reached the value 0.01. In all analyses, the first 25% trees from each run were discarded as burn-in.

3. Results

3.1 Molecular data and markers polymorphism

The data concerning sequence size (range) and aligned lengths to each dataset, number of variable positions in the alignments, parsimony informative characters (PI) are presented in Table 2.

Nine of the twelve regions (*trn*Q-*rps*16, *trn*H-*psb*A, *trn*L-*trn*F plus *trn*L intron, *psb*K-*psb*I, *mat*K, *rpo*B, ITS, *nad*1, *nad*4) were more informative including deletion,

insertion, transversion and transition that can help in the identification of species and clarify the relationship among and within sections *Viperella* and *Hydastylus* (Table S3 A-C). This group of plants showed higher number of transition than transversion for apomorphic characters in nuclear and chloroplast genome. It was not detected deletion or insertion in the nuclear genome, while the mitochondrial and chloroplast genomes showed a higher number of deletions than insertions. However, the insertion events would help in the identification of species. This is the case of *S. vaginatum* subsp. *vaginatum* with an insertion of 9 bp with a sequence duplicated (TAATAAGGT) in *psb*K-*psb*I (200-208 bp). *S. marchio* also display an insertion of 9 bp (ATGTAAAG) in *trn*Q-*rps*16 region (919-927 bp). Accessions belonging to *Hydastylus* and *Viperella* shared an insertion of 10 bp with a sequence duplicated (TGCTTTCAGA) in *nad*1 (516-525 bp) except for the species *S. weirii* and *S. alatum*. In the same way, these accessions shared deletions in the following regions: in *nad*1 region (469-501 bp) except for *S. alatum*; and in *nad*4 (903-906 bp) except for *S. palmifolium* subsp *palmifolium*.

3.2 Phylogenetic analyses

Phylogenetic trees resulting from combined dataset (COMB 3 and COMB4 – Fig 1 and Fig 2) showed better resolution than separated datasets (ITS, COMB1 and COMB2 – Fig. S1-S3). The results suggest that the sections *Hydastylus* and *Viperella* correspond to a single natural group of plants. However, the main groups were formed among species belonging to the same section.

Within the section *Hydastylus* all accessions of *Sisyrinchium antemeridianum* grouped together in well supported trees in the MP, ML and BI analyses using combined dataset (COMB3 and COMB4). *S. densiflorum* is related to *S. nidulare* with well supported values in MP, ML and BI analyses using all combined datasets. *S.*

densiflorum and *S. nidulare* share four synapomorphies in the nuclear and plastidial genome and together with *S. marchioides* share four synapomorphies in the mitochondrial genome (Table S3 A-C). These species also appeared related with high support (MP BS = 100, ML BS = 100 and BI PP= 1.0) in Chauveau et al. (2011), using combined datasets. Different accessions of *S. palmifolium* and even different accessions of the same subspecies (*S. palmifolium* subsp. *palmifolium*), do not cluster in the same lineage. Specifically, samples of *S. palmifolium, S. palmifolium* subsp. *palmifolium*, *S. palmifolium* subsp. *fuscoviride* and *S.* aff. *palmifolium* form a large group with high support in the MP (BS= 100), ML (BS= 100) and BI (PP= 0.97) trees using COMB4 dataset. However, the others accessions of *S. palmifolium* sp. and/or of *S. palmifolium* subsp. *palmifolium* are related to other species of the section *Hydastylus* or are splitted along the trees with low resolution in the different analyses presented here, indicating intraspecific polymorphism. *S. rectilineum* is related to *S. wettsteinii* with well support in the MP, ML and BI analyses using ITS and COMB4 dataset (Fig 2 and Fig S1). These relationship is also highly supported (MP BS = 87, ML BS = 93 and BI PP= 1.0) by Chauveau et al. (2011), using combined dataset.

Within the section *Viperella* one accession of *Sisyrinchium balansae* is related to *S. vaginatum* and to *S. vaginatum* subsp. *vaginatum* forming a group with internal node well supported in the MP, ML and BI analyses with combined dataset (COMB1 – Fig S2 and COMB3 - Fig 1). The other accessions of *S. balansae* are related to *S. parvifolium* and *S. restioides* also with high support in the MP, ML and BI analyses using COMB1 dataset. This last group was also observed, with high support (MP BS = 97, ML BS = 93 and BI PP= 1.0), by Chauveau et al. (2011). Furthermore, it was observed here that *S. balansae*, *S. parvifolium* and *S. restioides* shared three synapomorphies in the *mat*K region and other two in *trn*Q-*rps*16 (Table S3 – A and B).

The polyploid species (*S. marchio*, *S. marchioides* and *S. weirii*) grouped into a single group, except one accession of *S. marchio*, with high support in the MP, ML and BI analyses using COMB1 dataset (plastidial genome data). *S. marchioides*, *S. marchio* and *S. weirii* shared two exclusive synapomorphies in the *trn*Q-*rps*16 region. All polyploid accessions and *S. parvifolium* form a clade in the MP and BI analyses using COMB4 dataset (Fig 2). Furthermore, polyploid species were related to other species when analyzed with ITS and COMB2 dataset, as discussed below about the putative polyploid parentage.

3.3 Putative polyploid parentage

For primary inference of polyploid ancestry, we focus on the results derived from supported phylogenetic trees (BS > 60 or PP > 0.60), obtained through different analyses as previously described, it was observed the relationships among diploid and polyploid species.

Although polyploids would be derived from other polyploids, we identify some diploid(s) as the potential progenitor(s). Figure 3 summarize the inferred parentage of each polyploid.

4. Discussion

In this study, molecular data were used to evidence phylogenetic relationships among and within the sections *Hydastylus* and *Viperella* and to provide an insight into the evolutionary history of this group of species. The results strongly suggest that the two sections do not correspond to two clades but only a single natural group of plants in recent adaptive radiation. However, the main groups formed were among species of the same section. The phylogenetic analyses based on combined data of the three genomes

(COMB3 and COMB4) showed high resolution in MP trees and well supported groups. The markers that showed more molecular variation and parsimony informative characters for this group of plants were: the intergenic spacers *trn*L-*trn*F plus intron *trn*L and *rpo*B-*trn*C, followed by the nuclear ITS and mitochondrial DNA intron nad5. Three of these markers (except ITS) were tested by the first time for this group of species and have provided increase in the number of variable sites in the analysis, in relation to work of Chauveau et al. (2011), and therefore higher resolution in the phylogenetic trees.

Low phylogenetic resolution in the clade corresponding to the sections *Hydastylus* and *Viperella* in the work realized by Chauveau et al. (2011) probably is attributed to the lack of informative DNA sequence variation due to recent divergence within the genus and the number of accessions by species used for both sections. According with Whittall et al. (2006) the potential for polymorphism within and among species will require larger sample sizes. Congruence between multiple gene genealogies will be sought to minimize the effects of lineage sorting. This method should provide the necessary variation to resolve the phylogeny of many species-level phylogenies for recently radiating in non-model taxa. In this work, the number of markers and accessions compared to the work of Chauveau et al. (2011) was larger and provided higher phylogenetic resolution for the group encompassing species classified on sections *Hydastylus* and *Viperella*, once that Chauveau et al., focused in the whole genus *Sisyrinchium*.

Topology in the phylogenetic trees showed incongruences among the different genomes (mtDNA, cpDNA and nuDNA). This is probably due to differences in the evolutionary rates of each genome and to the events of reticulation probably happened in this group. The topologies resulting from MP and probabilistic analyses (BI and ML)

are in agreement regarding the resolution of major clades. Differences among trees do not supported relationships among samples in terminal clades. Moreover, attempts at resolving the phylogenetic relationships among Sysirinchium species with non-coding plastid regions and nuclear ribosomal spacers (ITS) have revealed surprisingly low sequence variation (Chauveau et al. 2011). Therefore, in the case of this group of plants is a clear need to increase the number of markers and the use of a very extensive sampling. Two possible explanations for this pattern can be proposed: (1) species are subject to introgression and allopolyploid events and subsequent speciation in this group of plants, (2) the species in question have diverged recently and/or rapidly. Each of them is discussed below.

Reticulate evolution in *Sisyrinchium*. The conflicts observed between topologies inferred from chloroplast vs. nuclear DNA vs. mitochondrial sequences among the sections *Hydastilus* and *Viperella* could resulted from homoplasy, retention of ancestral character states in one genome, or reticulate evolution (hybridization or introgression) (Willyard et al. 2011; Majure et al. 2012). Incongruence between nrITS and plastid topologies were observed also in species of tribe Trimezieae (Lovo et al. 2012) suggesting evidences of hybridization/introgression cycles, important processes in the diversification of Iridaceae (Tang et al. 2010; Taylor et al. 2011; Souza-Chies et al. 2012; Harpek et al. 2013). Therefore, reticulate evolution appears to be the most likely explanation. This fact may be reinforced by field observations of sympatric polyploid and diploid species and among different species belonging to the same or different sections and difficulty in circumscribe species based on morphological evidences. Furthermore, the phylogenetic trees also reinforced the hypothesis of reticulate evolution showed by intraspecific variation, with different accessions of the same species grouped into different clades.

Recent divergence in *Sisyrinchium*. The expected molecular pattern for a recently derived group could include incomplete lineage sorting as well as species that have differentiated but have not been separated for long enough to form monophyletic groups. The results presented here are consistent with both of these expectations. Therefore, we suggest that the sections *Hydastylus* and *Viperella* are in recent adaptive radiation. This event could occur so quickly that species may not have time to accumulate neutral DNA variation in their genomes (nuclear, plastidial and mitochondrial) as observed in this study. In addition the presence of problematic accessions (e.g. *S.* aff. *vaginatum* new species; *S. palmifolium* ESC405 and *S. antemeridianum*) in the phylogenetic analyses is consistent with the expected pattern of ancestral polymorphism associated with recently diverged lineages (Funk and Omland 2003).

Genetic diversity in this group of plants

Sisyrinchium palmifolium showed large genetic diversity with their accessions being related to other species of the section *Hydastylus* in different groups rather than grouping together. This can be due to the widespread distribution of the species in South America that can be resulted in a wide morphological variability with differences in plant size, habit (clump or rizomatoza), leaf morphology and inflorescence (in number of flowers and branches). On the other hand, *S. antemeridianum* that have restricted distribution being endemic to the Highlands of Southern Brazil (Aita et al. 2013) showed all accessions grouped together in the phylogenetic trees. This difference may be because endemic plant species with limited geographic distributions tend to have lower levels of genetic variation than species with more widespread distributions due to the effects of either directional selection promoting adaptation to local environments or random processes such as inbreeding, genetic bottlenecks, or drift acting in small

populations. Isolation may further reduce levels of genetic diversity within plant populations and enhance differences among them due to restricted gene flow (Hamrick et al., 1992). According with Lavergne et al (2004), reproductive traits of endemic species indicate a lower investment in pollen and seed production. This may be a consequence of the history of population isolation and persistence in fragmented cliff and rocky habitats (Orians 1997), and may also have favored genetic isolation from related species (Jain 1976), as observed here with *S. antemeridianum.*

Putative polyploid parentage

Leitch and Bennett (1997) have suggested that the evolutionary potential of a polyploid depends on a number of factors associated with the formation of the polyploid and with genetic divergence between the parents; unfortunately, the factors involved in the origin and establishment of polyploids in nature are largely unknown (Ramsey and Schemske 1998). The inferred patterns of polyploid origins proposed in this study (Fig.3) reveals a number of interesting features. First, the tetraploid *S. marchioides* can retain components of divergent genome of maternal lineages (*S. densiflorum*, *S. nidulare*, *S. marchio* and *S. weirii*) and divergent components of nuclear genome of paternal lineages (*S. alatum* and *S. parvifolium*) representing different putative combinations of diploid, tetraploid and hexaploid progenitors. *S. marchioides* is morphologically very similar to *S. alatum*, *S. marchio* and *S. weiri*. Basically differs by the presence of lower sphates in comparison with *S. alatum* and *S. marchio* and by larger size and the branches more divided in comparison with *S. weirii*. There are indications that tetraploid species *S. marchioides* has allopolyploid origin, given its simultaneous sympatric occurrence with the other diploid species in Rio Grande do Sul, where it is the only polyploid species occurring. Second, the tetraploid *S. marchio* can retain components of divergent genomes of maternal genetic lineages (*S. marchioides*

and *S. weirii*), and components of divergent nuclear genomes of paternal lineages (S. *parvifolium*, *S. restioides* and *S.* cf. *vaginatum*) representing different putative combinations of diploid, tetraploid and hexaploid progenitors. Third, the hexaploid *S. weirii* can retain divergent components of maternal genetic lineages (the two tetraploid *S. marchioides* and *S. marchio* and the diploid *S. alatum*) and one components of nuclear genome of paternal genetic lineages (*S. parvifolium*) representing different putative combinations among tetraploid and diploid species. Morphologically *S. weirii* is characterized by its compact size compared to its most similar species (*S. marchio*, *S. alatum*, *S. marchioides*) are unlikely to be confused with other species. *S. weirii* have relatively short branches topped by spathes and large flowers, roots and internodes very short. Chukr and Capellari Jr. (2003) proposed the synonymization for the species *S. parvifolium*, *S. balansae* and *S. weirii* under the taxon *S. vaginatum*, considering these four species as a single, due to the morphological plasticity found among populations. Corrêa (2011) using eight ISSR markers to analyse 13 species belonging to section *Viperella*, evidenced *S. weirii* with the highest number of polymorphic loci, possibly by the high degree of ancestral polymorphism considering this species an allopolyploid.

Based on these data, we suggest that the polyploidy species (*S. marchioides*, *S. marchio* and *S. weirii*) were independently derived from allopolyploidization events followed by divergent speciation. According with Mitton (1989), polyploids have increased heterozygosity, an attribute that may be beneficial. Polyploids also harbor higher levels of genetic and genomic diversity than was anticipated, with recurrent formation from genetically divergent diploid parents and possibly genome rearrangements contributing to the genetic diversity. This genetic diversity results in greater biochemical diversity, which also may be beneficial to the polyploid (Levin

1983). Finally, these genetic attributes may have ecological consequences as new interactions with other species, such as pollinators (Segraves and Thompson 1999).

Conclusion

We suggest that the sections *Viperella* and *Hydastylus* correspond to a group of plants in recent adaptive radiation and that the evolutionary history of the species has been impacted by allopolyploidy, introgression, and divergent allopatric species diversification. These events probably coincided with the last Pleistocene glaciations that have been cited as one of the most important recent diversification mechanisms of isolation followed by speciation mainly in the vegetation along the Atlantic cost. Our work provided evidence of synapomorphies that may help in the identification of species and evidence the relationship among and within sections *Viperella* and *Hydastylus*. These results would be used as a basis for future studies of DNA barcode for this group of plants. Moreover, it was the first time that hypothesis was raised about the origin of polyploid species (*S. marchio*, *S. marchioides* and *S. weirii*), related to diploid species of both sections. Therefore, we suggest that this is an ideal study group for investigating the evolutionary tempo of polyploidy and the complexities of reticulation and divergence, however more studies about the morphology, cytology and population genetic are needed to define more clearly the circumscription of taxonomic subdivision, gain a better understanding about the speciation patterns and provide evidences of interspecific gene flow.

Acknowledgments

This work received financial support provided by Brazilian Council for Research CNPq, CAPES/COFECUB and FAPERGS.

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TABLES AND FIGURES

Table 1: List of *Sisyrinchium* species from section *Hydastylus*, *Viperella* and outgroup, used in this study; with their respective, species, voucher, section, location and GenBank accession numbers for regions *rpo*C1, *rpo*B, *mat*K, *trn*H-*psb*A, *trn*Q-*rps*16, *trn*L-*trn*F plus intron *trn*L, *psb*K-*psb*I, *rpo*B-*trn*C, *nad*1-2/3, *nad*4-1/2, *nad*5-d/e and ITS. The asterisks correspond to the fragments used for each taxon in this study but still not added in GenBank and N to the missing data.

Section	**Species**	**Voucher**	**Geographical origin**	***rpo*C1**	***rpo*B**	***mat*K**	***trn*H-*psb*A**	***trn*Q-*rps*16**	***trn*L-*trn*F plus intron *trn*L**	***psb*K-*psb*I**	***rpo*B-*trn*C**
Hydastylus	*S. antemeridianum* Aita & L. Eggers	ESC200	RS, São Francisco de Paula	*	N	N	*	N	*	N	*
Hydastylus	*S. antemeridianum* Aita & L. Eggers	ESC204	RS, Cambará do Sul	*	N	N	*	N	N	*	*
Hydastylus	*S. antemeridianum* Aita & L. Eggers	ESC213	RS, São Francisco de Paula	*	N	N	*	N	*	*	*
Hydastylus	*S. antemeridianum* Aita & L. Eggers	ESC570	RS, Bom Jesus	*	N	N	*	N	*	*	*
Hydastylus	*S. brasiliense* (Ravenna) Ravenna	ESC379	PR, Guarapuava	HQ606550	HQ606660	HQ606770	HQ606880	HQ606990	N	N	N
Hydastylus	*S. bromelioides* R.C.Foster	ESC382	PR, Mariópolis	N	N	N	*	N	*	*	*

Section	**Species**	**Voucher**	**Geographical origin**	***rpo*C1**	***rpo*B**	***mat*K**	***trn*H-*psb*A**	***trn*Q-*rps*16**	***trn*L-*trn*F plus intron *trn*L**	***psb*K-*psb*I**	***rpo*B-*trn*C**
Hydastylus	*S. bromelioides* R.C.Foster. subsp. *bromelioides*	ESC410	SC, Campo Belo do Sul	HQ606556	HQ606666	HQ606776	HQ606886	HQ606996	N	N	N
Hydastylus	*S. caetanum* Ravenna	ESC224	SC, Bom Jardim da Serra	HQ606537	HQ606647	HQ606757	HQ606867	HQ606977	N	N	N
Hydastylus	*S. densiflorum* Ravenna	ESC321	SC, Campo Alegre	HQ606540	HQ606650	HQ606760	HQ606870	HQ606980	N	N	N
Hydastylus	*S. nidulare* (Hand.–Mazz.) I.M. Johnst.	ESC240	PR, Pinhão	HQ606522	HQ606632	HQ606742	HQ606852	HQ606962	*	*	*
Hydastylus	*S. palmifolium* L. subsp. *fuscoviride*	R. & E. Heaton SIS19900	Argentina (cultivated)	HQ606524	HQ606634	HQ606744	HQ606854	HQ606964	N	N	N
Hydastylus	*S. palmifolium* L. subsp. *palmifolium*	ESC469	RS, Aceguá	*	N	N	*	N	*	*	*
Hydastylus	*S. palmifolium* L.	ESC482	RS, Capão do Leão	*	N	N	*	N	*	*	*
Hydastylus	*S. palmifolium* L.subsp. *palmifolium*	ESC586	RS, Porto Alegre -Morro Santana	N	N	N	*	N	*	N	N
Hydastylus	*S. palmifolium* L. subsp. *palmifolium*	AITA19	RS, Porto Alegre-Morro do Osso	N	N	N	*	N	*	N	N
Hydastylus	*S. palmifolium* L.	ESC567	RS, São Francisco de Paula	*	N	N	*	N	*	*	*
Hydastylus	*S. palmifolium* L.	ESC487	RS, São Gabriel	*	N	N	*	N	*	*	*
Hydastylus	*S. palmifolium* L.	ESC486	RS, São Lourenço do Sul	*	N	N	*	N	*	*	N
Hydastylus	*S. palmifolium* L.	ESC193	RS, Viamão	*	N	N	*	N	*	*	*

Section	Species	Voucher	Geographical origin	*rpo*C1	*rpo*B	*mat*K	*trn*H-*psb*A	*trn*Q-*rps*16	*trn*L-*trn*F plus intron *trn*L	*psb*K-*psb*I	*rpo*B-*trn*C
Hydastylus	*S. palmifolium* L.	ESC206	RS, Viamão	N	N	N	*	N	*	N	*
Hydastylus	*S. aff. palmifolium* L.	ESC310	SC, Alto Arroio	*	N	N	*	N	*	*	*
Hydastylus	*S. palmifolium* L. subsp. *palmifolium*	ESC320	SC, Campo Alegre	*	N	N	*	N	*	*	*
Hydastylus	*S. palmifolium* L. subsp. *palmifolium*	Aita & Eggers 14	SC, Campo Alegre	N	N	N	*	N	*	N	N
Hydastylus	*S. aff. palmifolium* L.	ESC231	SC, Curitibanos	*	N	N	*	N	*	*	*
Hydastylus	*S. palmifolium* L.	ESC405	SC, Santa Cecília	*	N	N	*	N	*	*	*
Hydastylus	*S. palmifolium* L. subsp. *palmifolium*	Chauveau H099020	Unknown origin cultivated in USP	HQ606559	HQ606669	HQ606779	HQ606889	HQ606999	*	*	*
Hydastylus	*S. rectilineum* Ravenna	ESC284	RS, São Lourenço do Sul	*	N	N	*	N	*	*	*
Hydastylus	*S. rectilineum* Ravenna	ESC332	PR, Castro	HQ606542	HQ606652	HQ606762	HQ606872	HQ606982	N	N	N
Hydastylus	*S. wettsteinii* Hand.-Mazz.	ESC250	SC, Três Barras	HQ606539	HQ606649	HQ606759	HQ606869	HQ606979	N	N	N
Viperella	*S. alatum* Hook.	ESC232	SC, Irani	HQ606536	HQ606646	HQ606756	HQ606866	HQ606976	N	N	N
Viperella	*S. alatum* Hook.	ESC239	PR, Bituruna	*	N	N	*	N	*	*	*
Viperella	*S. balansae* Baker	ESC364	PR, Ponta Grossa	HQ606548	HQ606658	HQ606768	HQ606878	HQ606988	N	N	N
Viperella	*S. balansae* Baker	ESC560	RS, São Pedro do Sul	N	N	N	*	N	*	*	N

Section	Species	Voucher	Geographical origin	*rpo*C1	*rpo*B	*mat*K	*trn*H-*psb*A	*trn*Q-*rps*16	*trn*L-*trn*F plus intron *trn*L	*psb*K-*psb*I	*rpo*B-*trn*C
Viperella	*S. cf. marchioides* Ravenna	ESC406	SC, Santa Cecília	HQ606554	HQ606664	HQ606774	HQ606884	HQ606994	N	N	N
Viperella	*S. marchio* (Vell.) Steud.	ESC318	SC, Campo Alegre	*	N	N	*	N	*	*	*
Viperella	*S. marchio* (Vell.) Steud.	ESC407	SC, Santa Cecília	HQ606555	HQ606665	HQ606775	HQ606885	HQ606995	N	N	N
Viperella	*S. marchioides* Ravenna	ESC157	RS, Cambará do Sul	*	N	N	N	N	*	*	*
Viperella	*S. parvifolium* Baker	ESC 237	SC, Herciópolis	HQ606571	HQ606681	HQ606791	HQ606901	HQ607011	N	N	N
Viperella	*S. parvifolium* Baker	ESC464	RS, São Francisco de Paula	N	N	N	*	N	*	*	*
Viperella	*S. restioides* Spreng.	ESC252	SC, Correia Pinto	HQ606534	HQ606644	HQ606754	HQ606864	HQ606974	*	*	*
Viperella	*S. aff. vaginatum* Spreng.	ESC383	PR, Mariópolis	N	N	N	*	N	*	*	*
Viperella	*S. vaginatum* Spreng. subsp. *vaginatum*	ESC263	RS, Mostardas	HQ606572	HQ606682	HQ606792	HQ606902	HQ607012	*	*	*
Viperella	*S. cf. vaginatum* Spreng.	ESC476	RS, Capão do Leão	N	N	N	*	N	*	*	*
Viperella	*S. weirii* Baker	ESC248	PR, Palmeira	HQ606535	HQ606645	HQ606755	HQ606865	HQ606975	*	*	*
-	*Sisyrinchium sp.*	ESC278	RS, São Lourenço do Sul	N	N	N	*	N	*	*	*
Scirpeocharis	*S. fiebrigii* I.M.Johnst.	ESC 325	PR, Tijucas do Sul	HQ606541	HQ606651	HQ606761	HQ606871	HQ606981	N	N	N
Scirpeocharis	*S. fiebrigii* I.M.Johnst.	ESC352	PR, Ponta Grossa	*	N	N	*	N	*	*	*

Table 1: Continuation

Section	Species	Voucher	Geographical origin	*nad*1-2/3	*nad*4 1/2	*nad*5 d/e	ITS
Hydastylus	*S. antemeridianum* Aita & L. Eggers	ESC200	RS, São Francisco de Paula	*	N	*	*
Hydastylus	*S. antemeridianum* Aita & L. Eggers	ESC204	RS, Cambará do Sul	N	N	*	*
Hydastylus	*S. antemeridianum* Aita & L. Eggers	ESC213	RS, São Francisco de Paula	*	N	*	*
Hydastylus	*S. antemeridianum* Aita & L. Eggers	ESC570	RS, Bom Jesus	N	N	*	*
Hydastylus	*S. brasiliense* (Ravenna) Ravenna	ESC379	PR, Guarapuava	HQ607208	HQ607318	N	HQ607099
Hydastylus	*S. bromelioides* R.C.Foster	ESC382	PR, Mariópolis	N	N	*	*
Hydastylus	*S. bromelioides* R.C.Foster. subsp. *bromelioides*	ESC410	SC, Campo Belo do Sul	HQ607214	HQ607324	N	HQ607105
Hydastylus	*S. caetanum* Ravenna	ESC224	SC, Bom Jardim da Serra	HQ607195	HQ607305	N	HQ607086
Hydastylus	*S. densiflorum* Ravenna	ESC321	SC, Campo Alegre	HQ607198	HQ607308	N	HQ607089
Hydastylus	*S. nidulare* (Hand.–Mazz.) I.M. Johnst.	ESC240	PR, Pinhão	HQ607180	HQ607290	*	HQ607071
Hydastylus	*S. palmifolium* L. subsp. *fuscoviride*	R. & E. Heaton SIS19900	Argentina (cultivated)	HQ607182	HQ607292	N	HQ607073
Hydastylus	*S. palmifolium* L. subsp. *palmifolium*	ESC469	RS, Aceguá	N	N	*	N
Hydastylus	*S. palmifolium* L.	ESC482	RS, Capão do Leão	N	N	*	N
Hydastylus	*S. palmifolium* L.subsp. *palmifolium*	ESC586	RS, Porto Alegre - Morro Santana	N	N	N	N
Hydastylus	*S. palmifolium* L. subsp. *palmifolium*	AITA19	RS, Porto Alegre-Morro do Osso	N	N	N	N
Hydastylus	*S. palmifolium* L.	ESC567	RS, São Francisco de Paula	N	N	N	*
Hydastylus	*S. palmifolium* L.	ESC487	RS, São Gabriel	N	N	*	*
Hydastylus	*S. palmifolium* L.	ESC486	RS, São Lourenço do Sul	N	N	*	*
Hydastylus	*S. palmifolium* L.	ESC193	RS, Viamão	N	N	*	*

Section	Species	Voucher	Geographical origin	*nad*1-2/3	*nad*4 1/2	*nad*5 d/e	ITS
Hydastylus	*S. palmifolium* L.	ESC206	RS, Viamão	*	N	*	*
Hydastylus	*S. aff. palmifolium* L.	ESC310	SC, Alto Arroio	N	N	*	*
Hydastylus	*S. palmifolium* L. subsp. *palmifolium*	ESC320	SC, Campo Alegre	N	N	*	*
Hydastylus	*S. palmifolium* L. subsp. *palmifolium*	ESC650	SC, Campo Alegre	N	N	N	*
Hydastylus	*S. aff. palmifolium* L.	ESC231	SC, Curitibanos	*	N	*	*
Hydastylus	*S. palmifolium* L.	ESC405	SC, Santa Cecília	N	N	*	*
Hydastylus	*S. palmifolium* L. subsp. *palmifolium*	Chauveau H099020	Unknown origin cultivated in USP	HQ607217	HQ607327	*	HQ607107
Hydastylus	*S. rectilineum* Ravenna	ESC284	RS, São Lourenço do Sul	N	N	*	*
Hydastylus	*S. rectilineum* Ravenna	ESC332	PR, Castro	HQ607200	HQ607310	N	HQ607091
Hydastylus	*S. wettsteinii* Hand.-Mazz.	ESC250	SC, Três Barras	HQ607197	HQ607307	N	HQ607088
Viperella	*S. alatum* Hook.	ESC232	SC, Irani	HQ607194	HQ607304	N	HQ607085
Viperella	*S. alatum* Hook.	ESC239	PR, Bituruna	N	N	*	*
Viperella	*S. balansae* Baker	ESC364	PR, Ponta Grossa	HQ607206	HQ607316	N	HQ607097
Viperella	*S. balansae* Baker	ESC560	RS, São Pedro do Sul	N	N	*	*
Viperella	*S. cf. marchioides* Ravenna	ESC406	SC, Santa Cecília	HQ607212	HQ607322	N	HQ607103
Viperella	*S. marchio* (Vell.) Steud.	ESC318	SC, Campo Alegre	N	N	*	*
Viperella	*S. marchio* (Vell.) Steud.	ESC407	SC, Santa Cecília	HQ607213	HQ607323	N	HQ607104
Viperella	*S. marchioides* Ravenna	ESC157	RS, Cambará do Sul	N	N	*	*
Viperella	*S. parvifolium* Baker	ESC 237	SC, Herciópolis	HQ607229	HQ607339	N	HQ607119
Viperella	*S. parvifolium* Baker	ESC464	RS, São Francisco de Paula	N	N	*	*
Viperella	*S. restioides* Spreng.	ESC252	SC, Correia Pinto	HQ607192	HQ607302	*	HQ607083

Section	**Species**	**Voucher**	**Geographical origin**	***nad*1-2/3**	***nad*4 1/2**	***nad*5 d/e**	**ITS**
Viperella	*S. aff. vaginatum* Spreng.	ESC383	PR, Mariópolis	N	N	N	*
Viperella	*S. vaginatum* Spreng. subsp. *vaginatum*	ESC263	RS, Mostardas	HQ607230	HQ607340	*	HQ607120
Viperella	*S. cf. vaginatum* Spreng.	ESC476	RS, Capão do Leão	N	N	*	*
Viperella	*S. weirii* Baker	ESC248	PR, Palmeira	HQ607193	HQ607303	*	HQ607084
-	*Sisyrinchium sp.*	ESC278	RS, São Lourenço do Sul	N	N	*	*
Scirpeocharis	*S. fiebrigii* I.M.Johnst.	ESC 325	PR, Tijucas do Sul	HQ607199	HQ607309	N	HQ607090
Scirpeocharis	*S. fiebrigii* I.M.Johnst.	ESC352	PR, Ponta Grossa	N	N	*	N

Table 2. Comparison among nuclear, plastid, mitochondrial and combined datasets from *Sisyrinchium*.

Data Partition	Taxa number used	Size range (bp)	Aligned length (bp)	Conserved Characters (bp)	Variable characters (bp)	Parsimony informative characters (PI)
cpDNA						
*trn*L-*trn*F + intron *trn*L	34	404 - 704	721	598	118	69 (58%)
*psb*A-*trn*H	46	430-528	564	493	36	24 (67%)
*psb*K-*psb*I	28	218-382	398	357	27	10 (37%)
*rpo*B-*trn*C	28	290-558	585	451	121	51 (42%)
*rpo*C1	36	200-483	530	432	85	5 (6%)
*rpo*B	18	472	472	467	5	1 (20%)
*mat*k	18	1015	1015	998	17	3 (18%)
*trn*Q-*rps*16	18	1035-1050	1070	1019	33	14 (42%)
COMB1 (cpDNA spacers regions)	46	-	3308	2301	953	182 (19%)
mtDNA						
*nad*1	22	760-1618	1624	1603	21	4 (19%)
*nad*4	18	1660-1664	1664	1654	10	6 (60%)
*nad*5	29	383-820	840	749	81	27 (33%)
COMB2 (mtDNA regions combined)	22	-	4123	4031	84	34 (40%)
nuDNA						
ITS	46	555-662	690	594	93	40 (43%)
COMB3 (ITS + *trn*L-*trn*F + intron *trn*L + *psb*A-*trn*H + *psb*K-*psb*I + *rpo*B-*trn*C + *nad*5)	34	-	4272	3697	575	206 (36%)
COMB4 (ITS + COMB1 + COMB2)	46	-	7162	6799	308	115 (37%)

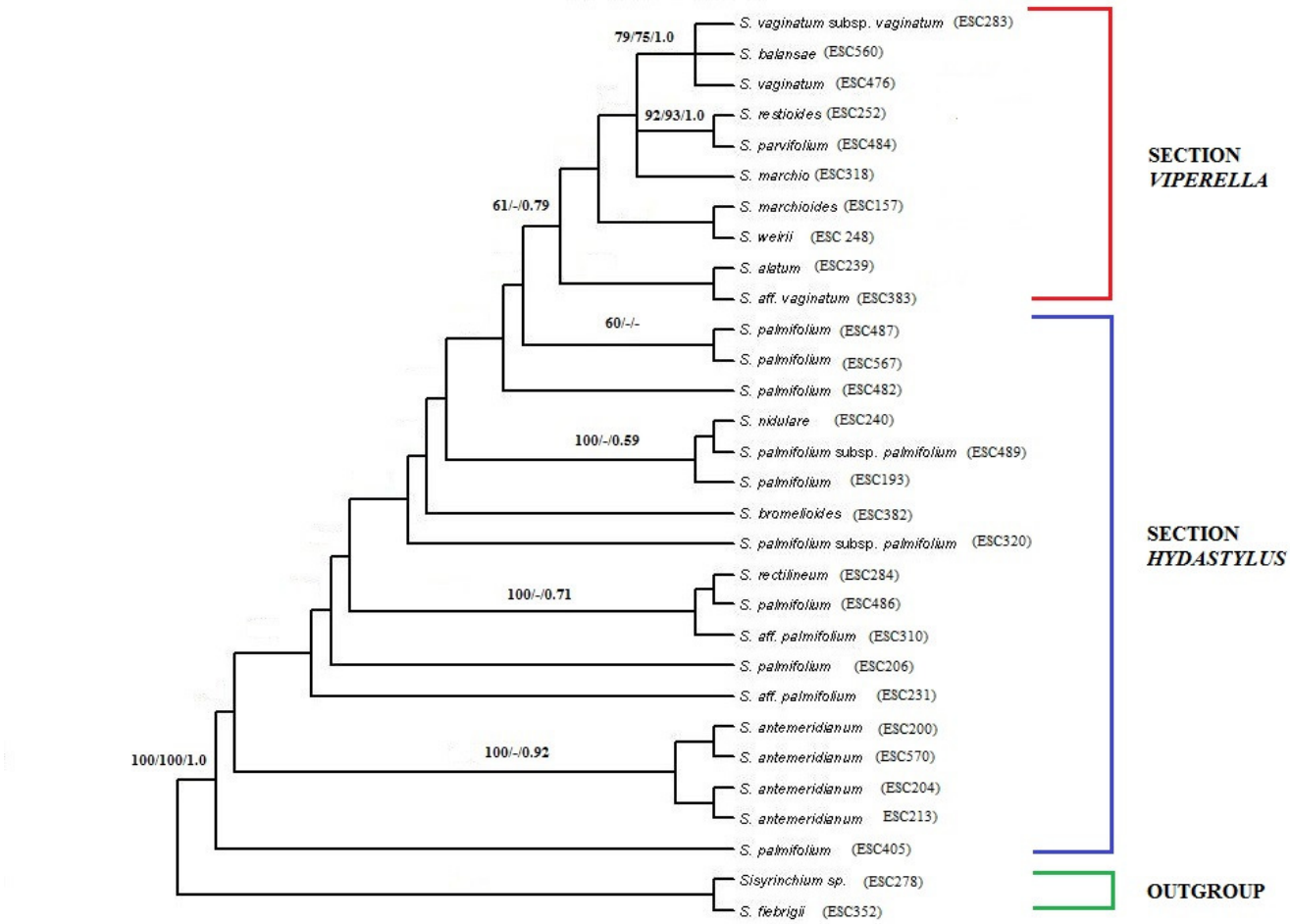

Figure 1 – Majority-rule consensus (50%) of 10 most parsimonious trees from ITS + *trn*L-*trn*F + intron *trn*L + *psb*A-*trn*H + *psb*K-*psb*I + *rpo*B-*trn*C + *nad*5 matrix (COMB 3). Support values along nodes correspond to Parsimony bootstrap/Likelihood bootstrap/Bayesian posterior probability. CI= 0.76 and RI= 0.67.

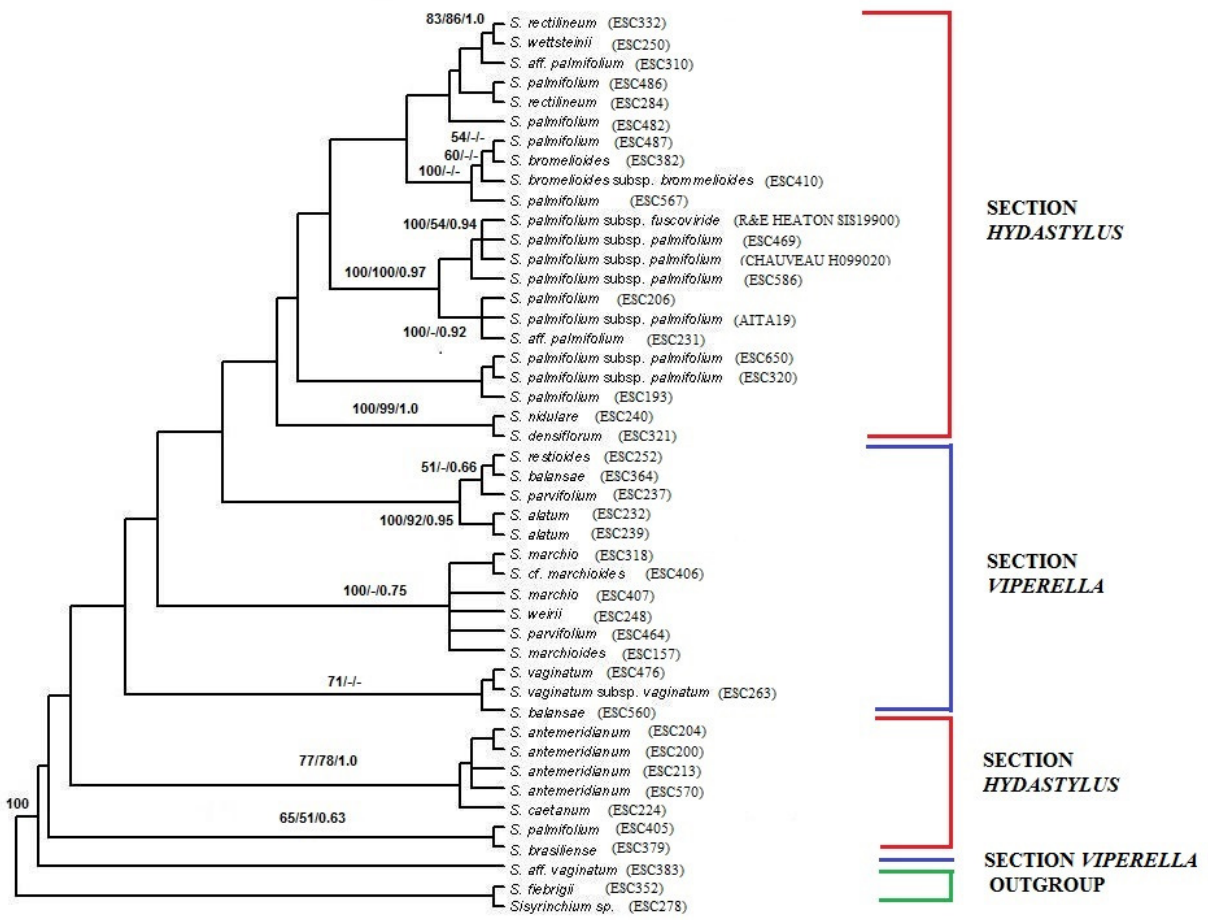

Figure 2 - Majority-rule consensus (50%) of 10 most parsimonious trees from ITS + COMB1 + COMB2 (COMB 4). Support values along nodes correspond to Parsimony bootstrap/Likelihood bootstrap/Bayesian posterior probability. CI= 0.81 and RI= 0.77.

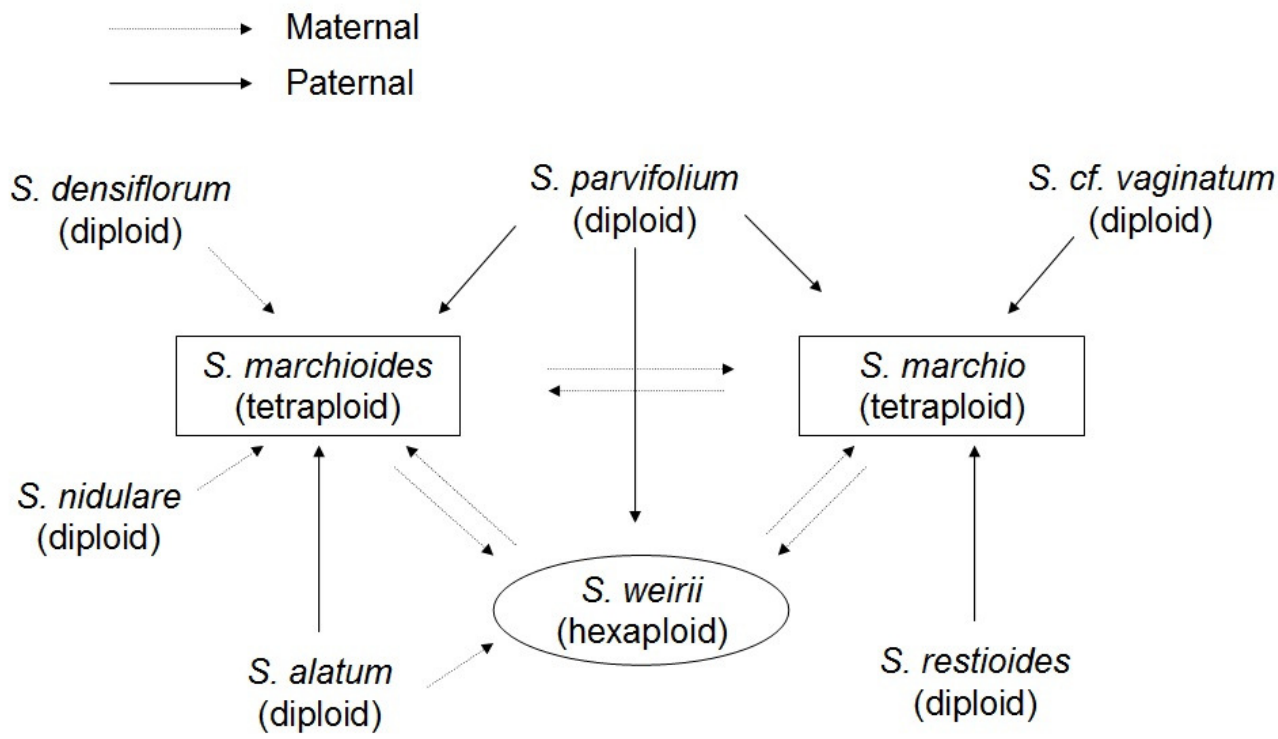

Figure 3: Inferred pattern of tetraploid origins from diploid, tetraploid and hexaploid progenitors and hexaploid origins from diploid and tetraploid progenitors. Maternal origins were based on findings from cpDNA and mtDNA trees. Paternal origins derived from the divergent placement of nuclear sequences in comparison to inferred maternal origins.

SUPPLEMENTARY DATA

Table S1. Primers used for amplifying and sequencing.

Primer name	Direction	PCR,sequencing	Primer sequence 5'-3'	Source
*rpo*C1	Forward	PCR,Seq	rpoC1-1f (GTGGATACACTTCTTGATAATGG)	http://www.kew.org/barcoding/protocols.html
	Reverse	PCR,Seq	rpoC1-3r (TGAGAAAACATAAGTAAACGGGC)	
*rpo*B	Forward	PCR,Seq	2f ATGCAACGTCAAGCAGTTCC	http://www.kew.org/barcoding/protocols.html
	Reverse	PCR,Seq	3r CCGTATGTGAAAAGAAGTATA	
*mat*K	Forward	PCR,Seq	matK-f1 (ATGGAAGAATTACAAGGATAT)	(Chauveau et al. 2011)
	Reverse	PCR,Seq		(Johnston and Soltis 1995)
	Forward	Seq	trnK-2r (AACTAGTCGGATGGAGTAG)	(Chauveau et al. 2011)
	Forward	Seq	matK-f2 (CATATAAACCAATTATCAAAC)	(Chauveau et al. 2011)
	Reverse	Seq	matK-f3 (CTTGTCTAAAGCTCAATTTTG)	(Chauveau et al. 2011)
	Reverse	Seq	matK-r1 (ATCCTGTACGATTGATACC) matK-r2 (AGTTTGATAATTGGTTTATATG)	(Chauveau et al. 2011)
*trn*H-*psb*A	Forward	PCR,Seq	psbA-f (GTTATGCATGAACGTAATGCTC)	(Sang et al. 1997)
	Reverse	PCR,Seq	trnH-r (CGCGCATGGTGGATTCACAAATC)	
*trn*Q-*rps*16	Forward	PCR,Seq	f – (GCGTGGCCAAGTGGTAAGGC)	modified from trnQ(UUG) (Shaw et al. 2007)
	Reverse		r – (GTTGCTTTCTACCACATCGTTT)	modified from rpS16x1 (Shaw et al. 2007)
*trn*L-*trn*F plus intron *trn*L	Forward	PCR,Seq	c B49317 (CGAAATCGGTAGACGCTACG)	(Taberlet et al. 1991)
	Reverse	PCR,Seq	f A50272 (ATTTGAACTGGTGACACGAG)	
*psb*K-*psb*I	Forward	PCR,Seq	psbK (TTAGCCTTTGTTTGGCAAG)	http://www.kew.org/barcoding/protocols.html
	Reverse	PCR,Seq	psbI (AGAGTTTGAGAGTAAGCAT)	
*rpo*B-*trn*C	Forward	PCR,Seq	rpoB-f (CKACAAAAYCCYTCRAATTG)	(Shaw et al. 2005)
	Reverse	PCR,Seq	trnC-r (CACCCRGATTYGAACTGGGG)	
*nad*1-2/3	Forward	PCR,Seq	nad1-2 (GCATTACGATCTGCAGCTCA)	(Demesure et al. 1995).
	Reverse	PCR,Seq	nad1-3 (GGAAGCCGATTAGTTTCTGC)	
*nad*4-1/2	Forward	PCR,Seq	*nad*4/1 (CAGTGGGTTGGTCTGGTAATG)	(Demesure et al. 1995).
	Reverse	PCR,Seq	nad4/2 (CAGTGGGTTGGTCTGGTAATG)	
*nad*5-d/e	Forward	PCR,Seq	nad5-4 (CCAATTTTTGGGCCAATTCC)	(Dumolin-Lapègue et al. 1997)
	Reverse	PCR,Seq	nad5-5 (CATTGCAAAGGCATAATGAT)	
ITS	Forward	PCR,Seq	92 (AAG GTT TCC GTA GGT GAA C)	(Desfeux and Lejeune 1996).
	Reverse	PCR,Seq	75 (TAT GCT TAA ACT CAG CGG G')	

Table S2. PCR profiles for DNA amplification: (1) initial denaturation; (2) number of cycles; (3) denaturation, annealing, and elongation steps for each cycle; (4) final elongation step. Temperature and duration are indicated for each step.

Locus	*PCR Profiles*
*rpo*C1	(1) 94°C-5 mn; (2) 40; (3) 94°C-1 mn, 58°C – 1mn, 72°C-1 mn; (4) 72°C-7 mn
*rpo*B	According Chauveau et al. 2011.
*mat*K	According Chauveau et al. 2011.
*trn*H-*psb*A	(1) 94°C-3 mn; (2) 35; (3) 94°C-1 mn, 56°C – 1mn, 72°C-1 mn; (4) 72°C-3 mn
*trn*Q-*rps*16	According Chauveau et al., 2011.
*trn*L-*trn*F plus intron *trn*L	(1) 94°C-3 mn; (2) 35; (3) 94°C-1 mn, 56°C – 1mn, 72°C-1 mn; (4) 72°C-3 mn
*psb*K-*psb*I	(1) 94°C-3 mn; (2) 35; (3) 94°C-1 mn, 56°C – 1mn, 72°C-1 mn; (4) 72°C-3 mn
*rpo*B-*trn*C	(1) 94°C-1 mn; (2) 40; (3) 94°C-30s, 56°C – 30s, 72°C-40s; (4) 72°C-5 mn
*nad*1-2/3	(1) 94°C-5 mn; (2) 40; (3) 94°C-1 mn, 56°C – 1mn, 72°C-1 mn; (4) 72°C-7 mn
*nad*4-1/2	According Chauveau et al. 2011.
*nad*5-d/e	(1) 94°C-5 mn; (2) 40; (3) 94°C-1 mn, 58°C – 1mn, 72°C-1 mn; (4) 72°C-7 mn
ITS	(1) 94°C-4 mn; (2) 35; (3) 94°C- 45s, 58°C – 1mn, 72°C-1:30 mn; (4) 72°C-2 mn

Table S3 A-C: Polymorphism showed by species of sections *Hydastylus* and *Viperella* in nuclear, chloroplast and mitochondrial markers.

Regions marked with gray correspond to synapomorphic characters.

Table S3 – A.

		Chloroplast Region																	
		*trn*Q-*rps*16												*trn*H-*psb*A					
Species	**Section**	**131**	**174-178**	**188**	**265**	**402**	**701**	**794**	**870**	**893**	**894**	**919-927**	**936-939**	**58**	**61**	**92**	**94-96**	**98**	**119-120**
S. antemeridianum	*Hydastylus*	N	N	N	N	N	N	N	N	N	N	N	N	C	A	T	TTT	G	deletion
S. brasiliense	*Hydastylus*	G	deletion	G	G	C	T	C	C	T	A		deletion	C	A	C	AAA	A	deletion
S. bromelioides	*Hydastylus*	N	N	N	N	N	N	N	N	N	N	N	N	T	G	T	TTT	G	deletion
S. bromelioides ssp. brommelioides	*Hydastylus*	T	deletion	G	G	C	T	C	C	A	T		deletion	T	G	T	TTT	G	deletion
S. caetanum	*Hydastylus*	G	deletion	G	G	C	T	C	C	T	A		deletion	C	A	T	TTT	G	deletion
S. densiflorum	*Hydastylus*	G	deletion	T	G	C	T	C	T	T	A		deletion	C	A	T	TTT	G	deletion
S. nidulare	*Hydastylus*	G	deletion	T	G	C	T	C	T	T	A		deletion	C	A	T	TTT	G	deletion
S. palmifolium ssp. fuscoviride	*Hydastylus*	T	deletion	G	G	C	T	C	C	A	T		deletion		G	T	TTT	G	deletion
S. palmifolium ssp. palmifolium	*Hydastylus*	T	deletion	G	G	C	T	C	C	A	T		deletion		G	T	TTT	G	deletion
S. palmifolium	*Hydastylus*	N	N	N	N	N	N	N	N	N	N	N	N	C (ESC 405 only)	A (ESC 405 only)	C (ESC 405 only)	AAA (ESC 405 only)	A (ESC 405 only)	deletion (except ESC 567 with TT)
S. aff. palmifolium	*Hydastylus*	N	N	N	N	N	N	N	N	N	N	N	N	T	G	T	TTT	G	deletion
S. rectilineum	*Hydastylus*	T	deletion	G	G	C	T	C	C	A	T		deletion	T	G	T	TTT	G	deletion
S. wettsteinii	*Hydastylus*	T	deletion	G	G	C	T	C	C	A	T		deletion	T	G	T	TTT	G	deletion
S. alatum	*Viperella*	G	deletion	G	G	C	T	C	C	T	A		deletion	C	G	T	TTT	G	TT
S. balansae	*Viperella*	G	deletion	G	G	T	C	C	C	T	A		deletion	C	A	T	TTT	G	CC

		Chloroplast Region																	
		trnQ-rps16												*trn*H-*psb*A					
S. cf. marchioides	*Viperella*	G	deletion	G	A	C	T	A	C	T	A		deletion	C	G	T	TTT	G	CC
S. marchio	*Viperella*	G	deletion	G	A	C	T	A	C	T	A	insertion (ATAGTAAAG)	deletion	C	G	T	TTT	G	CC
S. marchioides	*Viperella*	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
S. parvifolium	*Viperella*	G	deletion	G	G	T	C	C	C	T	A		deletion	C	G	T	TTT	G	deletion
S. restioides	*Viperella*	G	deletion	G	G	T	C	C	C	T	A		deletion	C	G	T	TTT	G	deletion
S. aff. *vaginatum* new species	*Viperella*	N	N	N	N	N	N	N	N	N	N	N	N	C	G	C	AAA	A	CC
S. vaginatum ssp. vaginatum	*Viperella*	G	deletion	G	G	C	T	C	C	T	A		deletion	C	A	T	TTT	G	CC
S. vaginatum	*Viperella*	N	N	N	N	N	N	N	N	N	N	N	N	C	A	T	TTT	G	CC
S. weirii	*Viperella*	G	deletion	G	A	C	T	A	C	T	A		deletion	C	G	T	TTT	G	CC
Sisyrinchium sp.	-	N	N	N	N	N	N	N	N	N	N	N	N	T	A	C	AAA	A	CC
S. fiebrigii	*Scirpeocharis*	G	TATTT	G	G	C	T	C	C	T	A		ATAT	T	A	C	AAA	A	CC

Table S3 – B.

		Chloroplast Region					
		*trn*L-*trn*F plus *trn*L intron	*psb*K-*psb*I	*mat*K			*rpo*B
Species	**Section**	**111-119**	**200-208**	**56**	**576**	**979**	**464**
S. antemeridianum	*Hydastylus*	GTTTTTAAA		N	N	N	N
S. brasiliense	*Hydastylus*	N	N	C	C	C	G
S. bromelioides	*Hydastylus*	deletion		N	N	N	N
S. bromelioides ssp. bromelioides	*Hydastylus*	N	N	C	C	C	A
S. caetanum	*Hydastylus*	N	N	C	C	C	G
S. densiflorum	*Hydastylus*	N	N	C	C	C	G
S. nidulare	*Hydastylus*	GTTTTTAAA		C	C	C	G
S. palmifolium ssp. fuscoviride	*Hydastylus*	N	N	C	C	C	G
S. palmifolium ssp. palmifolium	*Hydastylus*	deletion		C	C	C	G
S. palmifolium	*Hydastylus*	deletion (except ESC 405)		N	N	N	N
S. aff. palmifolium	*Hydastylus*	deletion		N	N	N	N
S. rectilineum	*Hydastylus*	deletion		C	C	C	G
S. wettsteinii	*Hydastylus*	N	N	C	C	C	G
S. alatum	*Viperella*	GTTTTTAAA		C	C	C	G
S. balansae	*Viperella*	GTTTTTAAA		T	T	T	A
S. cf. marchioides	*Viperella*	N	N	C	C	C	G
S. marchio	*Viperella*	GTTTTTAAA		C	C	C	A
S. marchioides	*Viperella*	GTTTTTAAA		N	N	N	N
S. parvifolium	*Viperella*	GTTTTTAAA		T	T	T	G
S. restioides	*Viperella*	GTTTTTAAA		T	T	T	G
S. aff. *vaginatum* new species	*Viperella*	GTTTTTAAA		N	N	N	N
S. vaginatum ssp. vaginatum	*Viperella*	GTTTTTAAA	TAATAAGGT (insertion of 9 bp with duplication of sequence)	C	C	C	G
S. vaginatum	*Viperella*	GTTTTTAAA		N	N	N	N
S. weirii	*Viperella*	GTTTTTAAA		C	C	C	G
Sisyrinchium sp.	-	G-TTTTAAA		N	N	N	N
S. fiebrigii	*Scirpeocharis*	G-TTTTAAA		C	C	C	G

Table S3 – C.

		Nuclear Region								Mitochondrial Region										
		ITS								*nad*1					*nad*4					
Species	**Section**	**125**	**191**	**195**	**226**	**385**	**440**	**512**	**558**	**478**	**479**	**480-484**	**496-501**	**516-525**	**721**	**896**	**903-906**	**971**	**1043**	**1252**	**1640**
S. antemeridianum	*Hydastylus*	C	G	C	T	A	A	C	C	A	G	TCAAG	deletion	TGCTTTCAGA (insertion with duplication of sequence)	N	N	N	N	N	N	N
S. brasiliense	*Hydastylus*	C	G	C	T	G	A	T	C	T	A	deletion	deletion	TGCTTTCAGA (insertion with duplication of sequence)	T	A	deletion	T	C	G	C
S. bromelioides	*Hydastylus*	C	G	C	T	G	A	T	C	A	G	TCAAG	deletion	TGCTTTCAGA (insertion with duplication of sequence)	N	N	N	N	N	N	N
S. bromelioides ssp. bromelioides	*Hydastylus*	C	G	C	T	G	A	T	C	A	G	TCAAG	deletion	TGCTTTCAGA (insertion with duplication of sequence)	T	A	deletion	T	C	G	C
S. caetanum	*Hydastylus*	C	G	C	T	A	A	T	C	A	G	TCAAG	deletion	TGCTTTCAGA (insertion with duplication of sequence)	T	A	deletion	T	C	G	C
S. densiflorum	*Hydastylus*	C	G	C	T	G	G	T	T	A	G	TCAAG	deletion	TGCTTTCAGA (insertion with duplication of sequence)	G	A	deletion	C	T	A	T
S. nidulare	*Hydastylus*	C	G	C	T	G	G	T	T	A	G	TCAAG	deletion	TGCTTTCAGA (insertion with duplication of sequence)	G	A	deletion	C	T	A	T
S. palmifolium ssp. fuscoviride	*Hydastylus*	C	G	C	T	G	A	T	C	A	G	TCAAG	deletion	TGCTTTCAGA (insertion with duplication of sequence)	T	A	deletion	T	C	G	C
S. palmifolium ssp. palmifolium	*Hydastylus*	C	G	C	T	G	A	T	C	A	G	TCAAG	deletion	TGCTTTCAGA (insertion with duplication of sequence)	T	C	TATC	C	C	G	C

		Nuclear Region								Mitochondrial Region										
		ITS								*nad*1					*nad*4					
S. palmifolium	*Hydastylus*	C	G	C	T	G	A	C	C	A	G	TCAAG	deletion	TGCTTTCAGA (insertion with duplication of sequence)	N	N	N	N	N	N	N
S. aff. palmifolium	*Hydastylus*	C	G	C	T	G	A	T	C	A	G	TCAAG	deletion	TGCTTTCAGA (insertion with duplication of sequence)	N	N	N	N	N	N	N
S. rectilineum	*Hydastylus*	C	G	C	T	G	A	C	C	A	G	TCAAG	deletion	TGCTTTCAGA (insertion with duplication of sequence)	T	A	deletion	T	C	G	C
S. wettsteinii	*Hydastylus*	C	G	C	T	G	A	C	C	A	G	TCAAG	deletion	TGCTTTCAGA (insertion with duplication of sequence)	T	A	deletion	T	C	G	C
S. alatum	*Viperella*	A	A	C	C	G	A	T	C	A	G	TCAAG	AGGGAG		T	A	deletion	T	C	G	C
S. balansae	*Viperella*	C	G	T	T	G	A	T	C	A	G	TCAAG	deletion	TGCTTTCAGA (insertion with duplication of sequence)	T	A	deletion	T	C	G	C
S. cf. marchioides	*Viperella*	A	G	T	T	G	A	T	C	T	A	deletion	deletion	TGCTTTCAGA (insertion with duplication of sequence)	G	A	deletion	C	T	G	T
S. marchio	*Viperella*	A	G	T	T	G	A	T	C	A	G	TCAAG	deletion	TGCTTTCAGA (insertion with duplication of sequence)	T	A	deletion	T	C	G	C
S. marchioides	*Viperella*	A	G	T	T	G	A	T	C	N	N	N	N	N	N	N	N	N	N	N	N
S. parvifolium	*Viperella*	C	G	T	T	G	A	T	C	T	A	deletion	deletion	TGCTTTCAGA (insertion with duplication of sequence)	T	A	deletion	T	C	G	C
S. restioides	*Viperella*	C	G	T	T	G	A	T	C	A	G	TCAAG	deletion	TGCTTTCAGA (insertion with duplication of sequence)	T	A	deletion	T	C	G	C
S. aff. *vaginatum* new species	*Viperella*	C	G	C	T	G	A	T	C	N	N	N	N	N	N	N	N	N	N	N	N

		Nuclear Region								Mitochondrial Region										
		ITS								*nad*1					*nad*4					
S. vaginatum ssp. vaginatum	*Viperella*	C	G	T	T	G	A	T	C	T	A	deletion	deletion	TGCTTTCAGA (insertion with duplication of sequence)	T	A	deletion	T	C	G	C
S. vaginatum	*Viperella*	C	G	T	T	G	A	T	C	A	G	TCAAG	deletion	TGCTTTCAGA (insertion with duplication of sequence)	N	N	N	N	N	N	N
S. weirii	*Viperella*	C	G	T	T	G	A	T	C	A	G	TCAAG	AGGGAG		T	A	deletion	T	C	G	C
Sisyrinchium sp.	-	C	G	C	T	A	A	T	C	N	N	N	N	N	N	N	N	N	N	N	N
S. fiebrigii	*Scirpeocharis*	C	G	C	T	A	A	T	C	A	G	TCAAG	AGGGAG		T	C	TATC	C	C	G	C

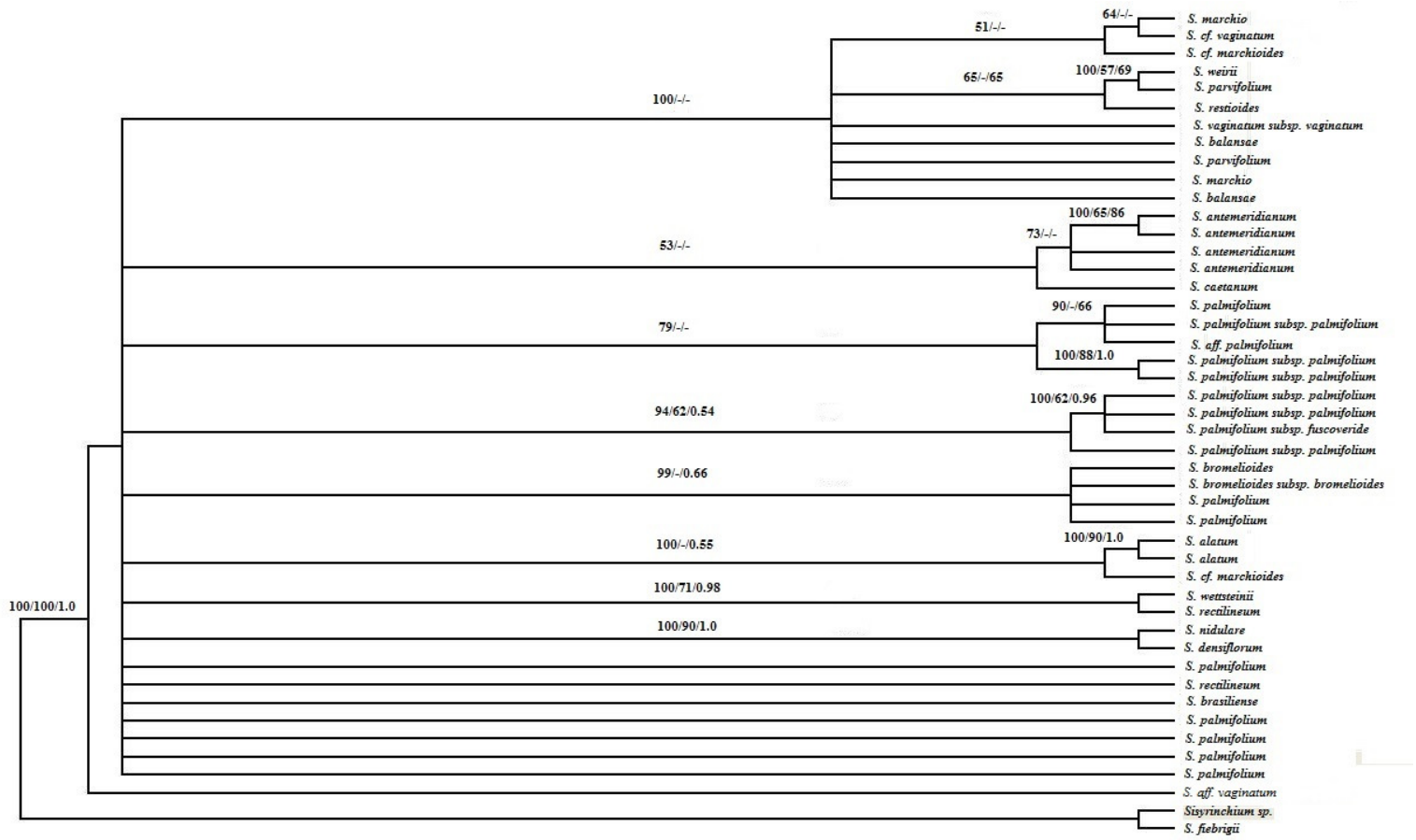

Figure S1 – Majority-rule consensus (50%) of 127 most parsimonious trees from ITS matrix. Support values along nodes correspond to Parsimony bootstrap/Likelihood bootstrap/Bayesian posterior probability. CI= 0.81 and RI= 0.79.

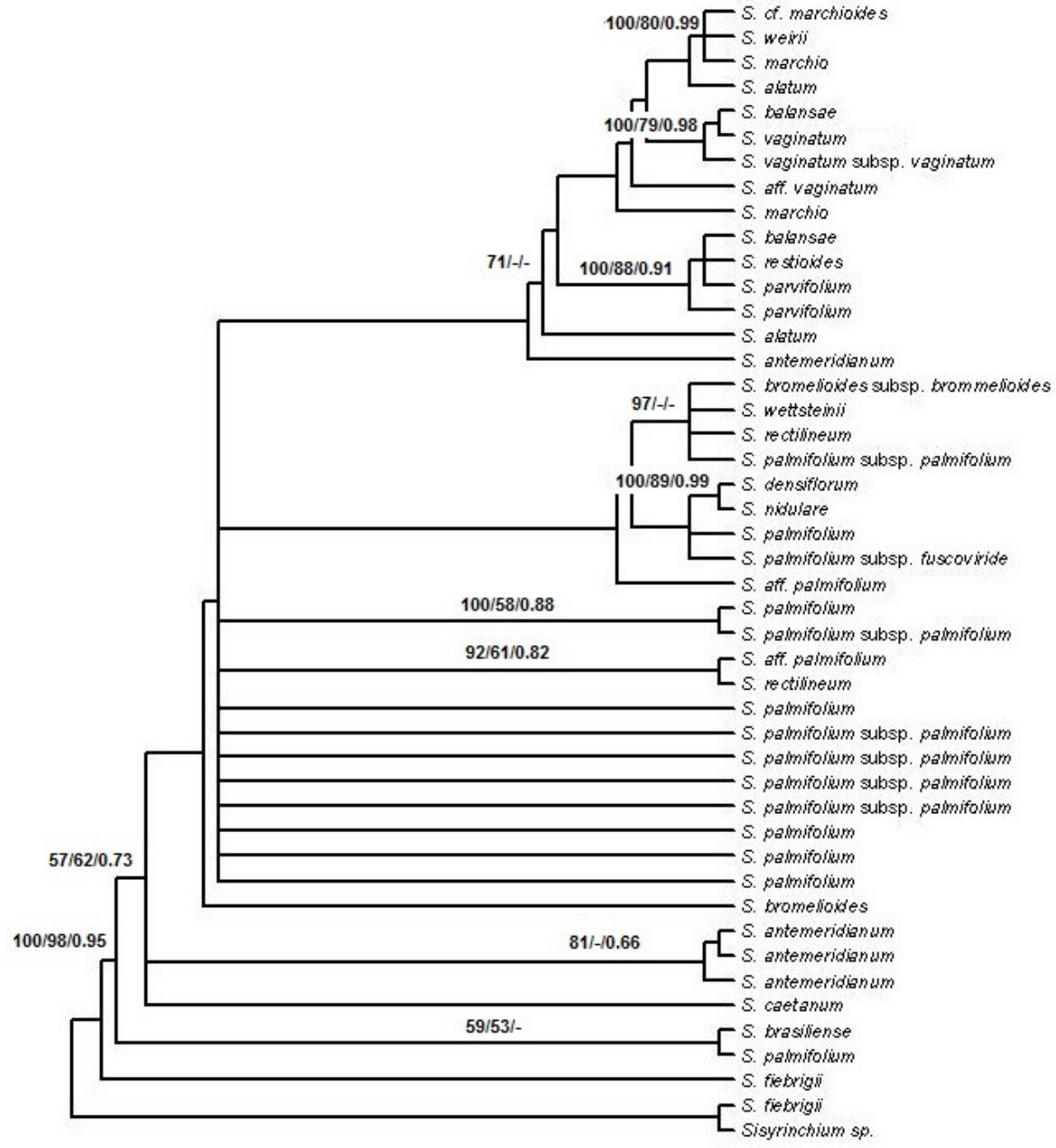

Figure S2 – Majority-rule consensus (50%) of 236 most parsimonious trees from cpDNA intergenic spacers matrix (COMB1). Support values along nodes correspond to Parsimony bootstrap/Likelihood bootstrap/Bayesian posterior probability. CI= 0.89 and RI= 0.76.

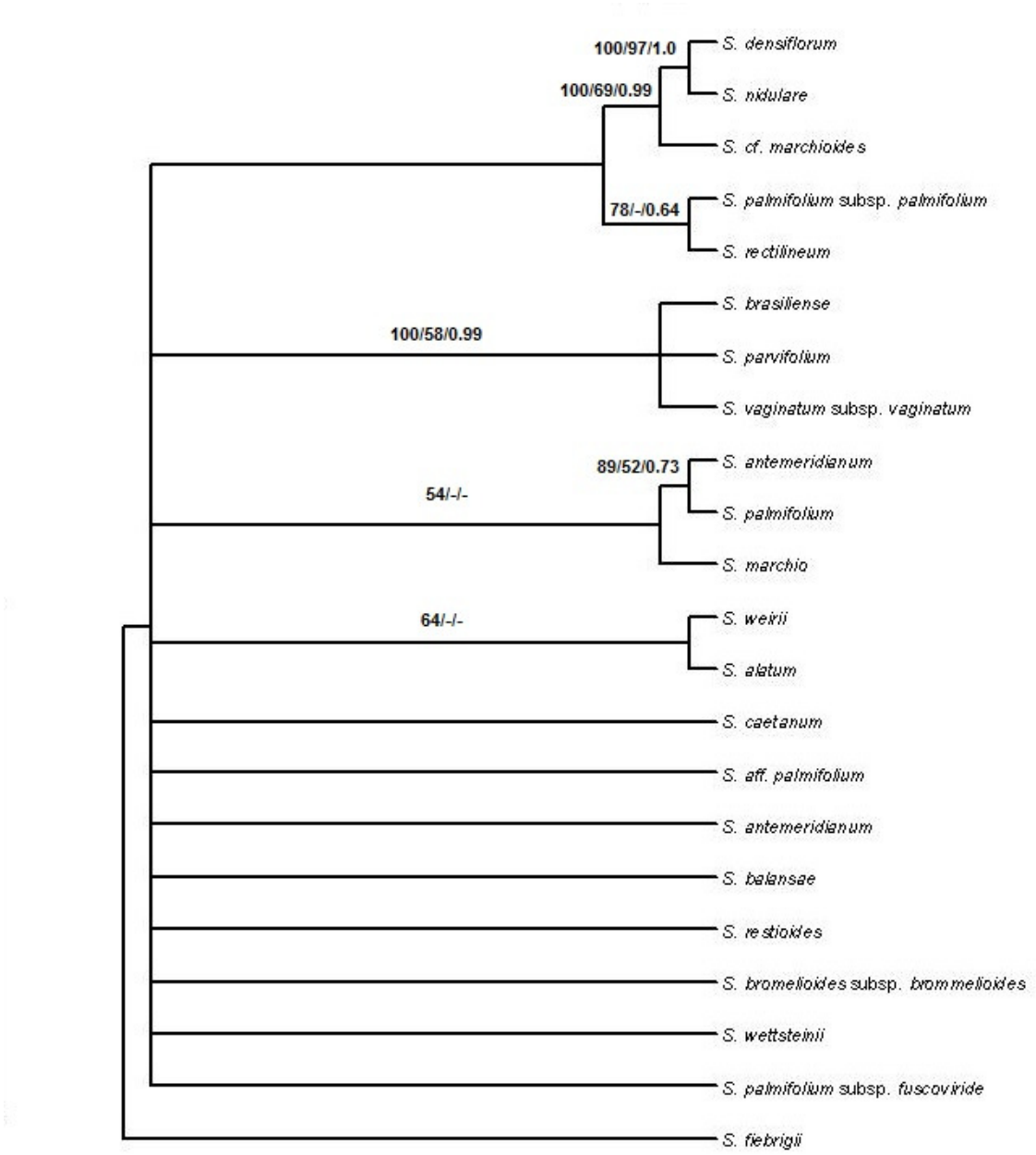

Figure S3 - Majority-rule consensus (50%) of 661 most parsimonious trees from mtDNA spacers regions matrix (COMB2). Support values along nodes correspond to Parsimony bootstrap/Likelihood bootstrap/Bayesian posterior probability. CI= 0.83 and RI= 0.71.

CAPÍTULO IV

Isolation, characterization and cross amplification of microsatellite loci in *Sisyrinchium palmifolium* (diploid) and *S. marchioides* (tetraploid) (Iridaceae)

A ser submetido para Revista Biocheimical Systematics and Ecology

Isolation, characterization and cross amplification of microsatellite loci in *Sisyrinchium palmifolium* (diploid) and *S. marchioides* (tetraploid) (Iridaceae)

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Abstract

Microsatellite markers were isolated in *Sisyrinchium palmifolium* and *S. marchioides*, species from southern Brazil threatened by habitat loss and fragmentation, which will be useful to assess the population genetic structure of these species. Sixteen microsatellite loci were developed from an enriched genomic library, and nine of these were amplified. A total of 22 individuals from three populations of *S. marchioides* and 20 individuals from six populations of *S. palmifolium* were used to evaluate SSR polymorphism. All nine loci were polymorphic, with four to fifteen alleles per locus. In *S. marchioides* populations the observed and expected heterozygosities ranged from 0.318 to 0.909 and from 0.147 to 0.906 respectively, while in *S. palmifolium* populations it ranged from 0.350 to 0.950 and from 0.211 to 0.806, respectively. The development of these microsatellite markers will contribute to investigate polyploidy origin, as well as relationship among species of the sections *Viperella* and *Hydastylus* of *Sisyrinchium* and genetic variability of these species. Cross-amplification in other Iridaceae species was more successful with high range in loci from *S. marchioides*, demonstrating the applicability of these microsatellite markers in other taxa.

Keywords:

Fingerprinting

Genetic diversity

Genotyping

Microsatellite

Plant variability

1. Introduction

Sisyrinchium palmifolium L. and *Sisyrinchium marchioides* Spreng belong to Iridaceae family. South America has been suggested as the center of origin and distribution of these species (Chauveau et al., 2011). Both species are perennials herbs, with yellow flowers without elaiophores, whose only floral resource is pollen (Cocucci and Vogel, 2001). They are appreciated as ornamental plants and are also threatened by habitat loss and fragmentation. *Sisyrinchium palmifolium* is a diploid species classified in the section *Hydastylus* by Ravenna (2000) while *S. marchioides* is tetraploid, belonging to the section *Viperella* (Ravenna, 2002). These species appear in the same clade in the phylogeny of the genus *Sisyrinchium* (Chauveau et al., 2011) and have high morphological similarity concerning flowers traits, which can be attributable to a recent adaptive radiation of *Sisyrinchium* (Goldblatt, 1982). Knowledge about the patterns of diversity and gene flow among species in this clade is essential to understand the genetic consequences of the variation in mating systems or pollinator behavior and to address other questions concerning population genetic studies.

The aim of this study was to develop a set of polymorphic microsatellites (simple sequence repeats; SSR) markers for the closely related species *S. palmifolium* and *S. marchioides* to provide molecular tools to investigate polyploidy origin, as well as relationship between sections *Viperella* and *Hydastylus* and genetic variability of this group of plants.

In the present work, 16 loci (12 from *S. marchioides* and four from *S. palmifolium*) were isolated but only nine were polymorphic, being six from *S. marchioides* and three from *S. palmifolium* (Table 1).

2. Material and Methods

2.1 Sampling

A total of 22 individuals from three populations of *S. marchioides* (ESC 580, ESC 562 and LBC5) and 20 individuals from six populations of *S. palmifolium* (ESC 193, ESC 320, ESC 469, ESC 586, ESC 650 and AITA 19) were used to evaluate SSR polymorphism (Appendix A – Supplementary data).

2.2 Development of microsatellite loci and primers design

A microsatellite-enriched genomic library was performed for *S. palmifolium* and *S. marchioides*, using the methodology of biotinylated oligonucleotide sequences bound to streptavidin-coated magnetic particles, as described by Billotte et al. (1999). Genomic DNA was extracted from dried leaf material using the CTAB method (Doyle and Doyle, 1987) with few modifications. The total DNA was digested with *Rsa*I and enriched in (CT)8 and (GT)8 repeats. Enriched fragments were amplified by polymerase chain reaction (PCR) and then ligated into a pGEM T-easy vector (Promega) and transformed into competent XL1-blue *Escherichia coli* cells. Positive colonies (blue/white b-galactosidase selection) were tested by PCR to confirm the presence of inserts. Selected recombinant colonies, in a total of 96, were bi-directionally sequenced in an automated ABI PRISM 377 sequencer (Perkin Elmer, Applied Biosystems) using T7 and SP6 primers and BigDye terminator (version 3.1). Sequences were assembled and edited with Seqman (DNAStar). Repetitive regions were searched with the Simple Sequence Repeat Identification Tool (Temnykh et al., 2001). Of the selected clones, 17 contained microsatellite sequences with more than five repeats and proper flanking regions for

primer design. A total of 16 primer pairs flanking the repetitive regions were designed with the primer 3 program (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi).

For each SSR, the forward primer was synthesized with a 19 base-pair (bp) M13 tail (5′-CACGACGTTGTAAAACGAC-3′) following the method of Schuelke (2000), which involved three primers: a forward SSR-specific primer with the M13 tail at its 5′ end, a reverse locus-specific primer, and a universal M13 primer labelled with the fluorescent dyes, 6-FAM (Applied Biosystems).

2.3 Amplification conditions and genotyping

All PCR amplifications were performed in a Applied Biosystems Veriti thermocycler in 10 µL reactions containing: 30 ng DNA template, 1X Taq DNA buffer, 2 mM $MgCl_2$, 100 µm dNTPs, 5 pmol forward primer, 10 pmol reverse primer, 1 pmol universal M13 primer and 1 U Taq polymerase (CenBiot, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil). A 'touchdown' cycling program was used: 95 °C for 3 min, then 10 cycles of 94 °C for 30 s, 58 °C decreasing to 48 °C at 1 °C per cycle for 30 s, 72 °C for 30 s followed by 30 cycles of 94 °C for 30 s, 48 °C for 30 s, 72 °C for 30 s, followed by a final extension of 10 min at 72 °C. Loci were genotyped on an ABI 3730 DNA Analyzer Sequencer and sized against a LIZ molecular size standard using GeneMarker software (SoftGenetics).

2.4 Data analyses

Observed (Ho) and expected (He) heterozygosities, Shannon-Weiner diversity index (H') and Nei's measure of population differentiation (G_{ST}) were calculated with ATETRA (Van Puyvelde *et al*., 2009) for the tetraploid species and GENEPOP (Raymond and Rousset 1995; web version 4.2) and MSA (Dieringer and Schlötterer, 2003), for diploid species.

3. Results and Discussion

All six loci from *Sisyrinchium marchioides* showed a maximum of four alleles per individual, suggesting a tetraploid genome, while the three loci observed for *S. palmifolium* showed no more than two alleles per individual. A total of 57 alleles (Table 1) were identified using these SSR markers, ranging from four (VD2) to fifteen (VB6), to *S. marchioides*, and 28 alleles were identified, ranging from seven (PA5) to eleven (PD3), to *S. palmifolium.* The Shannon-Weiner diversity index values range from 0.317 to 2.408, with a mean of 1.602 for *S. marchioides*, and from 1.176 to 2.139, with a mean of 1.913 to *S. palmifolium*. The expected heterozygosity (He) for the polymorphic loci ranged from 0.147 to 0.906 with a mean of 0.678 to *S. marchioides,* and from 0.211 to 0.806, with a mean of 0.585 to *S. palmifolium*. The Gst value found for *S. marchioides* and *S. palmifolium* were 0.039 and 0.295, respectively, indicating a higher differentiation among accesses of *S. palmifolium* than those from tetraploid species which may be explained by the polyploid origin of *S. marchioides*. All nine loci were considered moderately to highly informative and, therefore, adequate for genetic studies.

Cross-species amplification with nine primers was tested for 19 species of *Sisyrinchium* (including *S. palmifolium* and *S. marchioides*), three species of *Calydorea* and three species of *Herbertia* (Table 2).

Six loci from *Sisyrinchium marchioides* have more successfully amplified among almost all species from all three genera than the primers from *S. palmifolium*, suggesting that the first markers can be useful for population genetic studies of many species of Iridaceae. These markers may also help to elucidate phylogenetic relationships wherever sequence data do not display sufficient resolution.

Acknowledgments

The authors would like to thank Camila Zanella and Márcia Goetze for analysis help; This work was supported by Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) and Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP).

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Tables

Table 1 Primer sequences and characteristics for microsatellite loci from *Sisyrinchium palmifolium* (P) and *S. marchioides* (V), including locus name, primer sequences, repeat type, number of alleles, allele size range, observed (Ho), expected (He) heterozygosity, Shannon-Weiner index (H') and Nei's measure of population differentiation (Gst).

Locus name	Primer sequence (5'-3')	Repeat motif	No of Alleles	Size range (bp)	Ho	He	H'	G_{ST}
S. marchioides								
VA5	F: CTTTGTCATCAGAGCTTCATGTGC*	(TA)6(GT)7	9	173-191	0.818	0.827	1.872	0.030
	R: GTCATCTCGTTGCAGCCACCT							
VA8	F: CCCAGGGGGAATTCAGAGTTAT*	(GT)22(GA)5(GT)4	12	206-232	0.909	0.906	2.408	0.048
	R: GGCTCCTATTGTCAGCTTGATG							
VB5	F: GTCCGCAAAAAGGTGAGCAAAT*	(CA)9	5	215-246	0.318	0.147	0.317	0.004
	R: CGGAACAATCGAACAAGTGACA							
VB6	F: CGATTGCCGATACGCCATAAA*	(GA)13	15	190-246	0.636	0.825	2.075	0.096
	R: ATGTTGTCTTCCCCCTCCATCA							
VC6	F: TGCTGTCAGTTGGGAATCATTG*	(GCT)5	12	186-225	0.909	0.845	2.039	0.039
	R: GGCAGCAGCATCAACAGCAT							
VD2	F: CAGTGAGGTCAGTGTTGCTT*	(TTA)6	4	145-162	0.545	0.521	0.905	0.018
	R: GTCTTGGTTGTGTTTTGTTG							
Average			-	-	0.689	0.678	1.602	0.039
S. palmifolium								
PA5	F: AAGCTCACAGCATACTTGATAAGG*	(GT)7	7	184-197	0.950	0.806	1.824	0.303
	R: TGTGAAGGAAGATGGATCTGAA							
PD3	F: CCACTTACTACCCCGAACTGTA*	(CA)7	11	179-213	0.850	0.740	2.139	0.287
	R: GGAGGAGTTGAGAAGACTTGTG							
PD6	F: CTGATTCGCAAGTGCATGA*	(CA)4CG(CA)16(TA)6	10	161-199	0.350	0.211	1.776	0.296
	R: CCCGGGATACAAAAACCTA							
Average			-	-	0.716	0.585	1.913	0.295

Table 2 Cross-amplification of nine microsatellites markers isolated from *S. marchioides* and *S. palmifolium* across 24 species of Iridaceae.

Sample	Species	VA5	VA8	VB5	VB6	VC6	VD2	PA5	PD3	PD6
ESC157	*S. marchioides* Ravenna	++	+	+	w	+	++	w	-	+
ESC200	*S. antemeridianum* Aita & L. Eggers	++	+	+	w	+	+	+	-	+
ESC239	*S. alatum* Hook.	++	+	+	+	+	+	+	+	w
ESC240	*S. nidulare* (Hand.–Mazz.) I.M. Johnst.	w	-	w	w	-	+	-	-	-
ESC248	*S. weirii* Baker	++	+	+	-	++	++	+	-	-
ESC252	*S. restioides* Spreng.	++	w	+	+	++	+	w	-	+
ESC263	*S. vaginatum* Spreng. ssp *vaginatum*	++	+	+	+	+	+	w	-	+
ESC278	*Sisyrinchium* sp.	-	-	-	-	-	w	-	-	-
ESC284	*S. rectilineum* Ravenna	++	++	+	+	+	+	++	++	+
ESC318	*S. marchio* (Vell.) Steud.	+	-	+	-	w	+	-	-	w
ESC331	*S. commutatum* Klatt	+	+	+	w	+	++	w	-	+
ESC352	*S. fiebrigii* I.M.Johnst.	w	-	+	-	w	+	-	-	w
ESC382	*S. bromelioides* R.C.Foster	w	+	+	w	+	+	-	+	w
ESC464	*S. parvifolium* Baker	++	+	+	+	+	++	w	-	+
ESC488	*Herbertia lahue* ssp *lahue* (Molina) Goldblatt	+	w	+	+	+	++	-	-	+
ESC520	*Herbertia quareimana* Ravenna	-	-	-	-	-	-	-	-	-
ESC521	*Herbertia* sp.	+	w	+	+	+	+	-	-	w
ESC560	*S. balansae* Baker	++	+	+	+	++	++	+	+	-
ESC586	*S. palmifolium* L.	++	++	-	-	+	+	++	++	+
ESC632	*Calydorea campestris* (Klatt) Baker	+	w	+	+	+	+	-	-	w
ESC678	*S. aff. luzula* Klotzsch ex Klatt	+	+	+	+	+	++	+	-	+
ESC684	*Calydorea crocoides* Ravenna	+	-	+	-	+	+	-	-	+
ESC688	*Calydorea crocoides* Ravenna	+	w	+	+	+	+	-	-	+
ESC689	*S. cf. luzula* Klotzsch ex Klatt	+	+	+	+	+	+	+	-	+
ESC690	*S. setaceum* Klatt	+	+	+	w	+	++	+	-	+

+, successful amplification with single band visualized; ++, successful amplification with more than one band visualized; w, weak amplification; -, unsuccessful amplification.

Supplementary data

Appendix A. Populations of *Sisyrinchium palmifolium* and *S. marchioides* collected in southern Brazil.

Species	Voucher	Geographic origin	Latitude and Longitude
S. palmifolium	ESC 193	RS, Viamão	30° 21' 53,0" S / 51° 01' 22,4" W
	ESC 320	SC, Campo Alegre	26° 10' 13,9" S / 49° 13' 59,6" W
	ESC 469	RS, Aceguá	31° 39' 00,8" S / 54° 09' 09,1" W
	ESC 586	RS, Porto Alegre	30º 03' 35,2" S / 51º 07' 28,1" W
	ESC 650	SC, Campo Alegre	26° 10' 16,5" S / 49° 14' 04,3" W
	AITA 19	RS, Porto Alegre	30° 07' 23.47" S / 51°13'36.73" W
S.marchioides	ESC 562	RS, Caxias do Sul	29º 04' 00,4" S / 50º 58' 31,5" W
	ESC 580	RS, São Francisco de Paula	30º 25' 25,9" S / 50º 30' 50,7" W
	LBC 05	RS, Pelotas	26° 10' 13,9" S / 49° 13' 59,6" W

CAPÍTULO V

Genetic relationships and variation in two closely related species of *Sisyrinchium*: *S. palmifolium* L. and *S. vaginatum* Spreng. (Iridaceae)

A ser submetido para Botanical Journal of the Linnean Society

Genetic relationships and variation in two closely related species of *Sisyrinchium*: *S. palmifolium* L. and *S. vaginatum* Spreng. (Iridaceae)

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ABSTRACT

Sisyrinchium palmifolium L and *Sisyrinchium vaginatum* Spreng (Iridaceae) are examples of recent adaptive radiation origins in Southern Brazil. The delimitation of these related species is problematic for taxonomists and phylogeneticists due to its high morphological similarity and to low nucleotide diversity found in plant genomes. However, up to now, the patterns of population diversity and gene flow among these two species were not evaluated. Here, microsatellites markers were used to assess patterns of genetic diversity, population structure and gene flow within and among populations of these species in southern Brazil. In this work high degrees of polymorphism (39 alelles) and genetic diversity were observed among individuals within populations. Our data indicate that the populations are structured and with low inbreeding coefficient. Both multilocus Bayesian approaches and *F* statistics indicates that populations of *Sisyrinchium vaginatum* subsp. *vaginatum* are more structured than *S. palmifolium* subsp. *palmifolium* populations. We support the hypothesis that many shared alleles among the populations are due introgression occurred in recent past, i.e. ancestral polymorphism. Our data provide little evidence of recent bottlenecks for these species when SMM was assumed ($P < 0.05$). Microsatellite data revealed population relationships congruent with traditional species delimitation. Despite that introgression contributed to large biodiversity in these species, the implementation of new conservation programs are necessary in southern biomes in Brazil to access the gene pool of these species.

Kew words: adaptative radiation - gene flow - genetic population - Iridaceae - microsatellites.

INTRODUCTION

Iridaceae has worldwide distribution, being well represented (about 2,000 species) in southern Africa and in temperate and highland South and Central America. This pattern is largely the result of the exceptional radiation that has occurred in the subfamilies Crocoideae and Iridoideae. *Sisyrinchium* L. is the largest genus (approx. 140 species) of tribe *Sisyrinchieae* (Iridoideae) extending throughout South and North American from Patagonia to Greenland (Goldblatt & Manning, 2008). This genus has large diversification and abundance in Brazil (58 species) and is characterized by high morphological similarity among closely related taxa due to the recent adaptive radiation process undergone by these species, resulting in the emergence of species complexes (Souza-Chies *et al.*, 2012).

Sisyrinchium palmifolium L. and *Sisyrinchium vaginatum* Spreng have been well characterized as examples of a recent adaptive radiation in Southern Brazil (Chaveau *et al.*, 2011). These species are classified in the section *Hydastylus* by Ravenna (2000) and section *Viperella* (Ravenna, 2002) respectively. Recent phylogenetic inference of *Sisyrinchium* (Chauveau *et al.*, 2011) showed that both sections grouped in a single clade suggesting that they belong to a single section. Both species are perennials herbs, present similarity in flowers traits, with yellow flowers without elaiophores (oil-producing structures), being the pollen the only floral reward to pollinators. In southern Brazil, they occur in the Atlantic Forest and Pampas (less common) Biomes and are usually found as natural sympatric populations or occur in sympatry with other related species, or isolated. These species have been also found as ornamental plants in gardens. Usually inhabit open areas, high-altitude grasslands, wet environments, edges of

montane forests, on slopes often among rocks (Heaton & Mathew, 1998; Freitas & Sazima 2003).

In the last 30 years the state of Rio Grande do Sul, Brazil, has shown a large decrease in the total area of natural grassland with a strong expansion of agricultural activities as well as decrease in the natural vegetation in Atlantic Forest Biome due urban expansion which has threatened these species of *Sisyrinchium* by habitat loss and fragmentation (Overbeck *et al.*, 2007).

Species delimitation within this group of plants has been a challenge for taxonomists and phylogeneticists, due the high morphological similarity among related species and to low polymorphism found in genomes (Chauveau *et al.*, 2011). Preliminary investigations, carried out separately, on the genetic variability within and among populations of *S. palmifolium* and *S. vaginatum* species using inter-simple sequence repeat (ISSR) markers indicated populations with high genetic structure, being the majority of the variation occurring within than among populations (Souza-Chies *et al.*, 2012 and Miz *et al.*, unpublished data). Our team has also examined phylogenetic relationships among species of sections *Hydastylus* and *Viperella* in southern Brazil and has observed evolutionary events of introgression (Miz *et al.*, unpublished data). However, up to now, the patterns of genetic diversity, population structure and gene flow between *S. palmifolium* and *S. vaginatum* species have not been evaluated. This knowledge is considered essential to understand their evolutionary processes, to define more clearly the circumscription of taxonomic subdivision, provide convincing evidence of interspecific gene flow, to identify ecological factors that may have affected population genetic structure and for the development of efficient conservation strategies.

Here we addressed the following questions: (I) What the patterns of genetic diversity, population structure and gene flow tell us about evolutionary history of these species? (II) Did interspecific gene flow (introgression) occur? (III) Does each species belong to a single lineage, and what is the likely biological significance of departures from congruence between the genetic data and traditional taxonomic groupings? (IV) How are structured the populations, and what are the practical implications for conservation?

MATERIALS AND METHODS

Population sampling

A total of 236 individuals from eight populations of *Sisyrinchium* (three populations of *S. palmifolium* subsp. *palmifolium*, four of *S. vaginatum* subsp. *vaginatum* and one of *S. rectilineum,* species allies to *S. palmifolium*) were sampled, which are distributed along the eastern region of the state of Rio Grande do Sul – Brazil, with altitude ranging from 7 to 835 m; Fig. 1 and Table 1. Fresh leaves were collected and stored in silica gel.

Molecular markers and genotyping assays

Eighteen microsatellite markers, isolated from *Sisyrinchium palmifolium* and *S. marchioides* (Miz *et al.*, unpublished data) and developed for the species *Sisyrinchium micranthum* (Tacuatiá *et al.*, 2012a), were tested for each analyzed taxon. However, polymorphisms were detected only with six primers that were used in this study. For molecular genotyping, total genomic DNA was extracted from silica-gel-dried leaves using a modified approach based on Doyle & Doyle (1987), and DNA was quantified using a Nanodrop Spectrophotometer.

All PCR amplifications were performed in a Applied Biosystems Veriti thermocycler in 10 µL reactions containing: 30 ng DNA template, 1X Taq buffer, 2 mM MgCl2, 100 µm dNTPs, 5 pmol forward primer, 10 pmol reverse primer, 1 pmol universal M13 primer labelled with one of the fluorescent dyes, FAM (Applied Biosystems, Foster City, CA, USA), and 1 U Taq polymerase (CenBiot, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil). A 'touchdown' cycling program was used: 95 °C for 3 min, then 10 cycles of 94 °C for 30 s, 58 °C decreasing to 48 °C at 1 °C per cycle for 30 s, 72 °C for 30 s followed by 30 cycles of 94 °C for 30 s, 48 °C for 30 s, 72 °C for 30 s, followed by a final extension of 10 min at 72 °C. Loci were genotyped on an ABI 3730 DNA Analyzer Sequencer and sized against a LIZ molecular size standard using GeneMarker software (SoftGenetics).

Statistical analyses

Genetic diversity - In order to characterize the microsatellite loci, the following statistics were calculated: (1) A - number of alleles; (2) Ho - observed heterozygosity and (3) He - expected heterozygosity under Hardy-Weinberg equilibrium (HWE) (Nei, 1978). All genetic diversity parameters were measured with the program MICROSATELLITE ANALYSER (MSA) (Dieringer & Schlötterer, 2003) and FSTAT 1.2 (Goudet, 1995). GENEPOP on Web, version 3.5 (Raymond & Rousset, 1995), was used to estimate the inbreeding coefficient F_{IS} (Weir & Cockerham, 1984), overall inbreeding coefficient F_{IT} and fixation index F_{ST} (Table S1 – in the Supplementary Data).

In addition, each locus was tested, using the same diversity parameters, in species level to explore whether genetic diversity can change due to locus-specific effects or rather due to systematic factors (Table S2).

Subsequently, the genetic diversity within each population was characterized using the following diversity parameters: allelic richness, within-population inbreeding coefficients F_{IS} and Shannon Index (I) (Table 2). All of these genetic diversity parameters were corrected for sample size in FSTAT and POPGEN Version 1.32 (Yeh *et al.*, 1999).

Indirect analysis of gene flow via F-statistics

Analysis of molecular variance (AMOVA) in GENALEX (Peakall & Smouse, 2006) was used to obtain F-statistics for microsatellites at different hierarchical levels using the species-level data. We tested the hierarchies 'among populations', 'among individuals' and 'within populations' for the entire dataset. Subsequently, separate AMOVA models were analyzed to test the distribution of genetic variance of each species. The significance of each F-statistic was tested through 9999 permutations in GENALEX at the appropriate hierarchical level.

The resulting microsatellites data of eight populations was analyzed and an unbiased genetic distance matrix (Nei, 1978) was generated by TFPGA (Tools for Population Genetic Analyses, version 1.3) (Miller, 1997) to construct an Unweighted Pair-Group Method Arithmetic (UPGMA) average topology, which computed 1,000 permutations and estimated the confidence limits of the dendrogram and after allowing a 1,000 replicate bootstrap test using the same program. Marker frequencies were estimated based on the Lynch & Milligan (1994) Taylor expansion estimate.

Bayesian genetic structure analysis

Bayesian analysis in STRUCTURE version 2.3.3 (Hubisz *et al.*, 2009) was used to obtain additional insights regarding gene flow and *Sisyrinchium* population

subdivision. The most likely number of populations (*k*) was estimated under the admixture model and correlated allelic frequencies with no prior information on population origin. To determine the most likely number of clusters (*k*), our data were conditioned on different values of *k* ranging from 1 to 10. Analyses were carried out under the admixture model assuming independent allele frequencies and using a burn-in period of 5,000, run length of 50,000, and 10 iterations per *k* to confirm stabilization of summary statistics (Pritchard *et al.*, 2000). To determine the most likely number of clusters (*k*), the results generated by STRUCTURE were subsequently analyzed with STRUCTURE HARVESTER version 0.6.92 (Earl & vonHoldt, 2012) according to the method of Evanno *et al.* (2005), which is based on an ad hoc measure of Δk that evaluates the second-order rate of change of the likelihood function with respect to *k*.

Tests of basic assumptions for interpreting F-statistics in terms of historical gene flow

In order to determine if the sampled *Sisyrinchium* populations are likely to meet the equilibrium conditions required for the interpretation of F-statistics in terms of historical gene flow, the possibility of founder effects due to recent colonization (bottlenecks) was tested using the 'sign test', 'Standardized differences test" and 'Wilcoxon sign-rank' test in the BOTTLENECK program (Piry *et al.*, 1999). Both tests are able to detect recent reductions in effective population size due to genetic bottleneck. The analyses were carried out both for the 'infinite allele model' (IAM) and for the 'stepwise mutation model' (SMM) recommended for microsatellite data. The detection of recent bottleneck may indicate that *Sisyrinchium* populations are not in equilibrium between gene flow and genetic drift, which would render difficult the indirect estimation of gene flow via F_{ST} (Whitlock, 1992).

To test the correlation between genetic and geographic distances in km among populations, the Mantel Test was performed using GeneAlex 6.41 (Peakall & Smouse, 2006) with 10,000 permutations.

RESULTS

Microsatellite loci diversity in *Sisyrinchium* populations

All six microsatellite loci were polymorphic in *Sisyrinchium* populations from southern Brazil, showing a whole of 39 alleles (Table S1). The number of alleles per locus ranged from three (PA5) to nine (VA8) (Table S1). At species level it was observed up to seven alleles per locus (PD3) in *S. palmifolium* subsp. *palmifolium* and up to eight alleles per locus (VA5) in *S. vaginatum* subsp. *vaginatum* (Table S2). The populations of *Sisyrinchium* showed high genetic structure (Table S1). In species level, the populations of *Sisyrinchium palmifolium* subsp. *palmifolium* showed moderate genetic structure while *S. vaginatum* subsp. *vaginatum* was highly structured (Table S2). The inbreeding coefficient was higher in populations of *S. vaginatum* subsp. *vaginatum* than in populations of *S. palmifolium* subsp. *palmifolium* being significant to all loci except VA8 to both species and VA5 and PD3 for *S. vaginatum* subsp. *vaginatum.* Heterozygote excess was significant to locus VA5, PA5 and L3 for *S. palmifolium* subsp. *palmifolium,* being the last two significant also for *S. vaginatum* subsp. *vaginatum* (Table S2).

Patterns of genetic variability across populations

Populations of *Sisyrinchium* exhibited high levels of genetic diversity (Table 2). Allelic richness ranged from 2.863 (POP 5 - *S. vaginatum* subsp. *vaginatum*) to 5.945 (POP 4 - *S. rectilineum*), whereas the Shannon index (I) ranged from 0.733 (POP 5 - *S. vaginatum* subsp. *vaginatum*) to 1.127 (POP 4 - *S. rectilineum*). The inbreeding

coefficient (F_{IS}) ranged among the distinct populations (Table 2) and was significant in all populations, except for populations POP 4 and POP 7 in which deviations from Hardy-Weinberg proportions were not significant. Heterozygote excess was significant for populations POP2 and POP3 (*S. palmifolium* subsp. *palmifolium*), and POP8 (*S. vaginatum* subsp. *vaginatum*) (Table S3). The sympatric POP1 and POP 5 showed the higher inbreeding index (Table 2). In the tests for recent population bottlenecks, we found significant deviation from the expected ratio (1:1) at mutation–drift equilibrium when I.A.M was assumed ($P< 0.05$) for populations POP2, POP3, POP4 and POP7, suggesting that a historical bottleneck or founder event may have occurred in these populations mainly due to heterozygosity excess observed (Table S3). However, in other populations and when S.M.M. was assumed ($P<0.05$) no significant deviation was observed for any population, which allow us to interpret population differentiation (F_{ST}) in terms of inter-population gene flow (Table S3). The Mantel correlation between geographic and genetic distance was not significant (data not shown), thus suggesting the absence of isolation by distance among these populations of *Sisyrinchium*, as previously observed for populations of *Sisyrinchium palmifolium* subsp. *palmifolium* (Miz *et al.*, unpublished data) and *Sisyrinchium micranthum* from southern Brazil using ISSR markers (Tacuatiá *et al.*, 2012b).

Patterns of population divergence and gene flow

Analysis of molecular variance (AMOVA) of *Sisyrinchium* species attributed a significant proportion of the genetic variance (20%) to the 'among populations' level, and a similar and significant proportion to the 'among individuals' (21%) level, whereas most of the variance resided within populations (59%, Table 3; all *P*-values <0.001). Separate AMOVA models for each species revealed that, for *S. palmifolium* subsp.

palmifolium, a significant proportion of the genetic variance (6%) to the 'among populations' level, and a higher and significant proportion to the 'among individuals' (14%) level, whereas most of the variance resided within populations (80%, Table 3; all *P*-values <0.001). In the case of *S. vaginatum* subsp. *vaginatum,* a significant proportion of the genetic variance (21%) at populations level was found, and a similar and significant proportion among individuals (26%) level, whereas most of the variance resided within populations (53%, Table 3; all *P*-values <0.001), similar to that resulted for the three species together. Individual F_{ST} estimates between pairs of populations of *S. palmifolium* subsp. *palmifolium*, *S. rectilineum* and *S. vaginatum* subsp. *vaginatum* ranged from 0.026 to 0.306 (Table 4; all *P*-values <0.001). Individual F_{ST} estimates between pairs of populations of *S. palmifolium* subsp. *palmifolium* only range from 0.026 to 0.071; between pairs of populations of *S. vaginatum* subsp. *vaginatum* only range from 0.092 to 0.311 (Table 4). The pairwise F_{ST} values translate into estimates of gene flow (Nm) ranging from 0.553 to 9.404 among all populations. Considering only *S. palmifolium* populations Nm range from 3.227 to 9.404, and to *S. vaginatum* populations only Nm range from 0.553 to 2.464 (Table 4).

The dendrogram produced by TFPGA (Fig 2) based on Nei's unbiased genetic distance presented two main clusters with low support, one comprising the population of *S. palmifolium* subsp. *palmifolium* (bootstrap value 72%) grouped to the population of *S. rectilineum* (bootstrap value 40%), both belonging to section *Hydastylus.* The other group was formed by populations of *S. vaginatum* subsp. *vaginatum* (bootstrap value 30%). The population POP8 was the most distant genetically being in the base of the dendrogram.

Bayesian genetic structure analysis identified K=5 clusters: cluster 1 (POP2 and POP3 – *S. palmifolium*); cluster 2 (POP1 and POP4 – *S. palmifolium* and *S.*

rectilineum); cluster 3 (POP5 and POP7 – *S. vaginatum*); cluster 4 (POP6 - *S. vaginatum*) and cluster 5 (POP8 – *S. vaginatum*) (Fig 3) which is in agreement with genetic distance observed among populations in the dendrogram (UPGMA) (Fig 2).

DISCUSSION

This study documented considerable variation at microsatellite loci within and among natural populations of *Sisyrinchium palmifolium* subsp. *palmifolium*, *S. rectilineum* and *S. vaginatum* subsp. *vaginatum* in southern Brazil. Major variations were observed within rather than among populations (Table 3). Genetic differentiation among *S. palmifolium* and *S. vaginatum* populations was very high, while among *S. palmifolium* and *S. rectilineum* was moderate, and among *S. vaginatum* and *S. rectilineum* was moderate to high (Table 4). Therefore, the data indicate structured populations and with low inbreeding coefficient. Both multilocus Bayesian approaches and *F* statistics indicates that populations of *Sisyrinchium vaginatum* subsp. *vaginatum* are more structured than *S. palmifolium* subsp. *palmifolium* (Fig 3 and Table 4).

Reticulate evolution (introgression) has been suggested to species of *Sisyrinchium* in recent phylogenetic study focused on the sections *Hydastylus* and *Viperella* (Miz *et al.*, unpublished data). The introgression has been pointed as an event that facilitate to adaptive radiation by providing the standing genetic variation required by ecological speciation (Seehausen, 2004). These data showed high levels of genetic diversity within populations and evidence of shared alleles among the populations belonging to the three species of *Sisyrinchium.* These alleles sharing can be explained by different processes: recent introgressive hybridization, the retention of ancestral polymorphism (incomplete lineage sorting), homoplasy (evolutionary convergence) or a combination of them. We support the hypothesis that many shared alleles is due introgression occurred in recent past, i.e. ancestral polymorphism. This can be

evidenced by the lack of correlation between genetic and geographic distance, and low interspecific gene flow shown among most populations of *S. palmifolium* subsp *palmifolium* and *Sisyrinchium vaginatum* subsp. *vaginatum* (Nm < 1 migrant per generation), mainly between the sympatric populations POP1 (*S. palmifolium*) and POP5 (*S. vaginatum*) (Table 4). *Sisyrinchium rectilineum* showed significant rate of gene flow with the populations of *S. palmifolium* and *S. vaginatum* (Nm > 1 migrant per generation). However, Bayesian admixture (Fig. 3) and genetic distance (Fig.2) showed *S. rectilineum* more related to *S. palmifolium* than to *S. vaginatum* populations, agreeing with previous morphological and phylogenetic data (Ravenna, 2000; Miz *et al.*, unpublished data).

Recent bottlenecks could strongly influence present-day genetic structure. However, in all populations, heterozygosities did not deviate significantly from expectations under SMM. According to various authors (Jin *et al.* 1996; Chakraborty *et al.* 1997; Ellegren, 2004; Caliebe *et al.*, 2010; Barthe *et al.*, 2012), SMM is the model that best describes the evolutionary dynamics of most SSR loci, and then analyses assuming this model are more accurate than those assuming the IAM. Therefore, our data provide little evidence of recent bottleneck for these species (Table S3). Among the populations that showed significant deviated mutation-drift equilibrium with IAM (POP2, POP3, POP4 and POP7) (Table S3), only the populations of *S. palmifolium* (POP 2 and POP3) and the population of *S. rectilineum* (POP4) shown also significant deviated of HWE through the analysis of inbreeding coefficient (F_{IS}). These data suggest that heterozygosity excess (Table 2 and Table S3) observed in these populations of section *Hydastylus*, may be result from a bottleneck or founder event. Recent work about genetic variability of *Sisyrinchium palmifolium* subsp. *palmifolium* population from southern Brazil using nine ISSR markers (Miz et. al., unpublished data) showed

also significant deviation from the expected ratio (1:1) at mutation–drift equilibrium, however when both SMM and IAM were assumed ($P < 0.05$). Furthermore, ISSR data indicated a heterozygosity deficiency, suggesting that a historical expansion may have occurred in the populations unlike that found here. This discrepancy in results may be due the different type of inheritance of markers, being ISSR dominant and SSR co-dominant.

The UPGMA tree allowed us to test genetic relationship among populations belonging to the three species: *S. palmifolium* subsp. *palmifolium*, *S. rectilineum* and *S. vaginatum* subsp. *vaginatum* (Fig. 2). Three trends are clearly visible from this analysis. Firstly, populations of *S. palmifolium* subsp. *palmifolium* group together with bootstrap support of 72%. Thus, microsatellite data provide an evidence of population relationship that is congruent with traditional taxonomic species' delimitation for these taxa (Ravenna, 2002). Second, *S. rectilineum* is closer to *S. palmifolium* than to *S. vaginatum* as already mentioned above. Third, populations of *S. vaginatum* subsp. *vaginatum* clustered together with low bootstrap support, except the population POP8. This can be explained because the population POP8, is located on the north coast of the state of Rio Grande do Sul, a region which has undergo strong impact in flora and fauna due urban expansion. In this work, the population POP8 was highly differentiated from populations of *S. palmifolium* and the populations POP5 and POP6 of *S. vaginatum*. The observed genetic diversity was high within this population (Table 2), showing low inbreeding coefficient and heterozygosity excess (F_{IS}= - 0.043). We suggest the following hypotheses regarding genetic differentiation of this population: First, the gene flow among this population and the populations POP4 and POP7 may be kept (Nm > 1) by migratory birds. Second, the significant gene flow among these populations may

reflect historical expansion events of this species, as suggested to populations of *S. palmifolium* subsp. *palmifolium* in Miz *et al.* (unpublished data).

This study revealed clear gene pool structuring in *S. palmifolium* subsp. *palmifolium*, *S. rectilineum* and *S. vaginatum* subsp. *vaginatum*. As indicated by AMOVA, genetic differences among populations explain 20%, 6% and 21% of the variation, considering all populations, *S. palmifolium* populations and *S. vaginatum* populations respectively. As expected for a predominantly outcrossing species, most genetic diversity was found within populations (Table 3). These finding are in agreement with previous surveys of variation attributed to populations of *S. palmifolium* subsp. *palmifolium* (65–66% variation - within populations – Miz *et al.*, unpublished data) and *S. vaginatum* (54% variation within populations – Souza-Chies *et al.*, 2012). This pattern of genetic variation in sympatric populations of *S. palmifolium* (POP1) and *S. vaginatum* (POP5) can be explained by the following: 1) Multiple pre- and postzygotic barriers may contribute to reproductive isolation between these species, as previously shown to other groups of plants (Widmer *et al.*, 2009); 2) Insect-pollinated perennial plants, such as *S. palmifolium* and *S. vaginatum*, are predicted to have self-incompatibility mechanisms to reduce the risk of genetic load (Barrett *et al.*, 1996; Morgan *et al.*, 1997).

Sisyrinchium vaginatum is listed as an endangered species (World Conservation Monitoring Centre 2000), according to the criteria described by the IUCN (International Union for Conservation of Nature), so this species deserve special attention. The great and significant genetic differentiation among populations of the three species, mainly among populations of *S. vaginatum* subsp. *vaginatum,* provided in this work, implies that efficient *in situ* or *ex situ* conservation strategies are needed based on a sufficient number of different accesses to represent the gene pool of each species. Therefore,

despite that introgression contribute to large biodiversity in these Iridaceae plants, the implementation of new conservation programs will need in south biomes to maintain access to gene pool of these species.

ACKNOWLEDGMENTS

We thank Adriana Aita, Lauís Brisolara Corrêa and Eudes Alves who kindly contributed with assistance in field trips, Camila Zanella and Márcia Goetze for their valuable help. We also thank the CNPq, CAPES/COFECUB, and FAPERGS for financial support.

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TABLES AND FIGURES

TABLES

Table 1. Sampled localities of *Sisyrinchium palmifolium* subsp. *palmifolium*, *S. rectineum* and *S. vaginatum* subsp. *vaginatum* in Rio Grande do Sul state from Brazil.

ID	Species	Voucher*	Locality	Latitude S	Longitude W	Altitude (m)
POP1	*S. palmifolium* subsp. *palmifolium*	ESC 586	RS, Porto Alegre (Morro Santana)	30º 03' 35.2"	51º 07' 28.1"	278
POP2	*S. palmifolium* subsp. *palmifolium*	ESC469	RS, Aceguá	31° 39' 00.8"	54° 09' 09.1"	172
POP3	*S. palmifolium* subsp. *palmifolium*	AITA 19	RS, Porto Alegre (Morro do Osso)	30° 7'20.00''	51°14'5.00''	143
POP4	*S. rectilineum*	ESC 284	RS, São Lourenço do Sul	31º 22' 22.2"	52º 05' 56.1"	9
POP5	*S. vaginatum* subsp. *vaginatum*	LBC 02	RS, Porto Alegre (Morro Santana)	30º 03' 35.2"	51º 07' 28.1"	278
POP6	*S. vaginatum* subsp. *vaginatum*	ESC 471	RS, Candiota	31° 31' 20.0"	53° 30' 40.1"	394
POP7	*S. vaginatum* subsp. *vaginatum*	ESC563	RS, Caxias do Sul	29º 04' 00.4"	50º 58' 31.5"	835
POP8	*S. vaginatum* subsp. *vaginatum*	ESC427	RS, Xangri-lá	29° 47' 08.7"	50° 02' 51.6"	7

* ESC – Lilian Ergges and Tatiana Souza-Chies; AITA – Adriana Aita; LBC – Lauís Brizolara Corrêa.

Table 2. Characterization of Rio Grande do Sul populations of *Sisyrinchium palmifolium* subsp. *palmifolium*, *S. rectilineum* and *S. vaginatum* subsp. *vaginatum* with six nuclear microsatellite markers, including sample size and the following diversity and breeding system parameters calculated: allelic richness, within-population inbreeding coefficients F_{IS} and Shannon Index (I).

Species	Population	n	Allelic richness	F_{IS}	Shannon Index (I)
S. palmifolium subsp. *palmifolium*	POP1	29	3.859	0.124	1.039
	POP2	30	3.154	-0.088	0.911
	POP3	30	3.346	-0.089	0.942
S. rectilineum	POP4	28	5.945	-0.016	1.127
S. vaginatum subsp. *vaginatum*	POP5	30	2.863	0.140	0.733
	POP6	30	3.402	0.063	0.964
	POP7	29	3.130	0.100	0.974
	POP8	30	3.193	-0.043	0.938

Departures of within-population inbreeding coefficients (F_{IS}) from HWE are indicated as $p<0.001$.

Table 3. Molecular variance (AMOVA) for the species *Sisyrinchium palmifolium* subsp. *palmifolium, S. rectilineum* and *S. vaginatum* subsp. *vaginatum* using three different hierarchical models: a three-level model including the three species, and separate three-level models for each species *S. palmifolium* subsp. *palmifolium* and *S. vaginatum* subsp. *vaginatum*.

Model	Partitioning	d.f.	SS	MS	Variation (%)	F-statistic
Three levels – three species	Among Pops	7	205,381	29,340	0,458	20%
	Among individuals	228	534,352	2,344	0,479	21%
	Within of population	236	327,000	1,386	1,386	59%
Three level – *S. palmifolium* subsp. *palmifolium*	Among Pops	2	17,390	8,695	0,111	6%
	Among individuals	86	179,706	2,090	0,278	14%
	Within of population	89	136,500	1,534	1,534	80%
Three level – *S. vaginatum* subsp. *vaginatum*	Among Pops	3	95,844	31,948	0,496	21%
	Among individuals	115	280,236	2,437	0,597	26%
	Within of population	119	148,000	1,244	1,244	53%

The significance of each F-statistic was tested through 9999 permutations at the appropriate hierarchical level. P< 0.001.

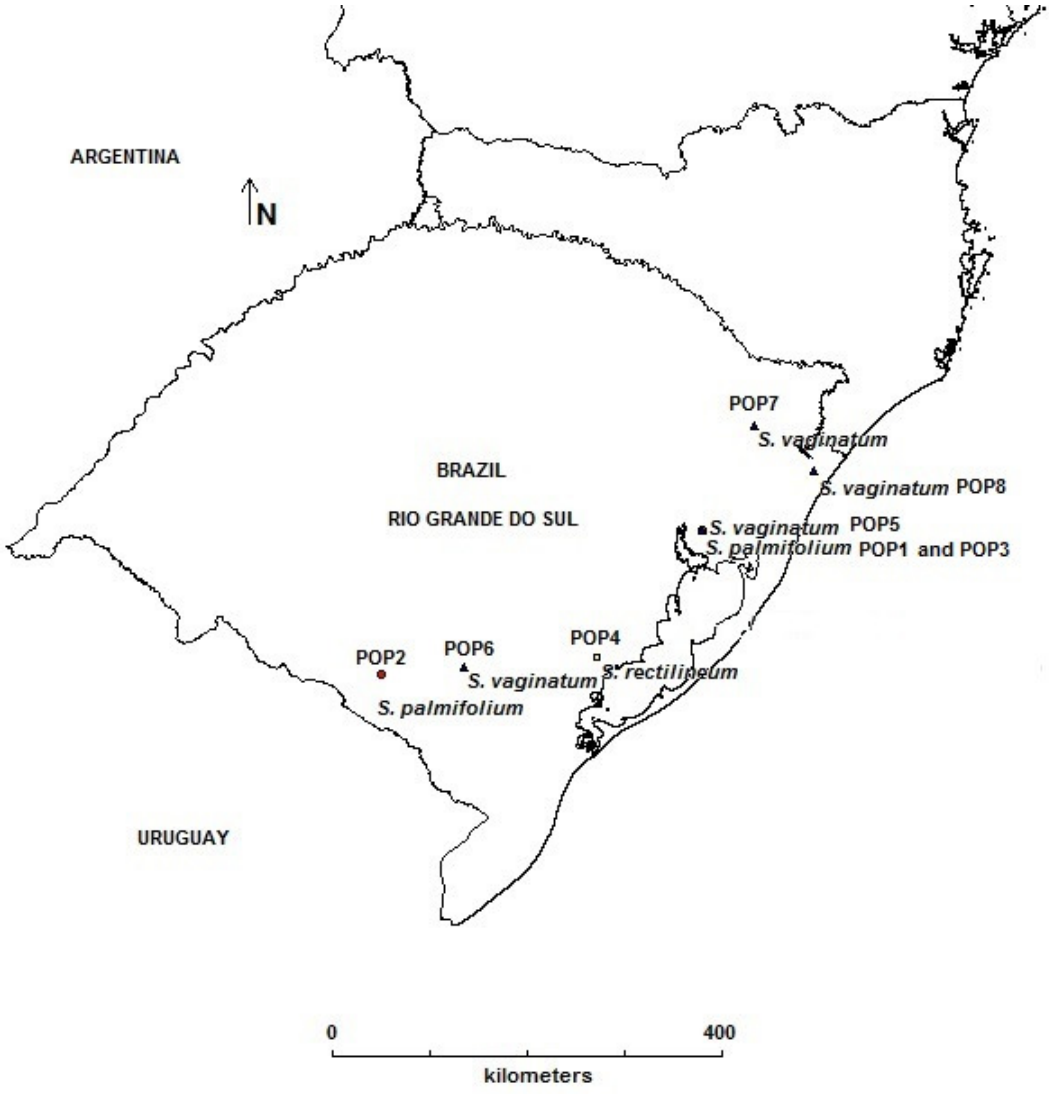
Table 4: Genetic divergence (F_{ST}; below diagonal) and gene flow (Nm; above diagonal) for populations pairs of *Sisyrinchium*.

	POP 1	POP2	POP3	POP4	POP5	POP6	POP7	POP8
POP1	-	3.247	3.227	1.897	0.567	0.810	0.814	0.736
POP2	0.071	-	9.404	1.551	0.665	0.916	1.128	0.610
POP3	0.076	0.026	-	1.758	0.654	0.926	1.200	0.570
POP4	0.116	0.139	0.125	-	1.612	1.284	1.975	1.176
POP5	0.306	0.273	0.277	0.134	-	0.964	2.464	0.618
POP6	0.236	0.214	0.213	0.163	0.206	-	1.246	0.553
POP7	0.235	0.181	0.172	0.112	0.092	0.167	-	1.160
POP8	0.254	0.291	0.305	0.175	0.288	0.311	0.177	-

All F_{ST} values were significant at the 0.001 level.

FIGURES

Figure 1: Distribution map of *Sisyrinchium* populations sampled in the South of Brazil.



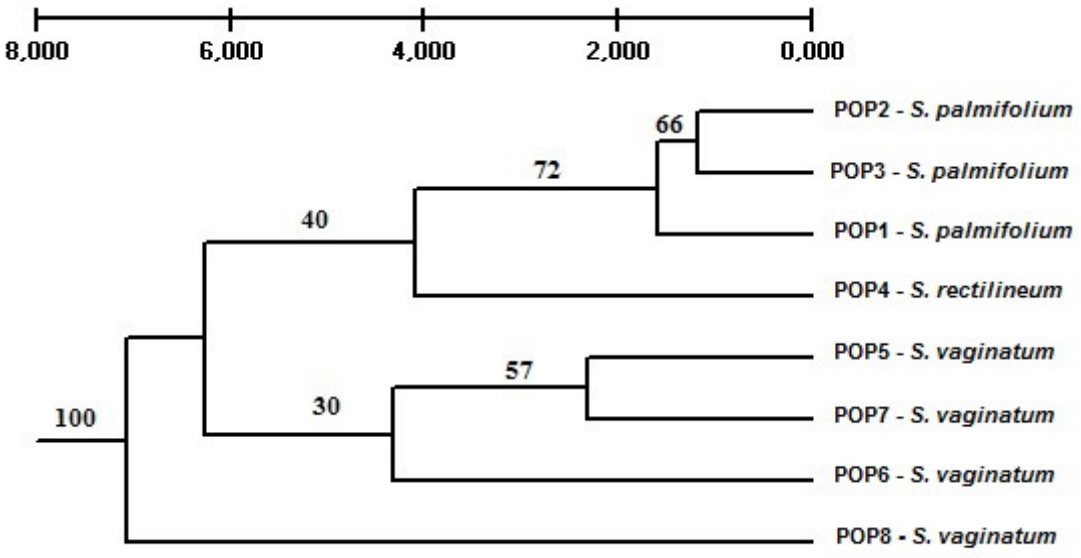


Figure 2. Unweighted pair-group method arithmetic average (UPGMA) based on Nei (1978) genetic distance (including bootstrap support values in percentages) using TFPGA software for all populations of *Sisyrinchium* in southern Brazil. A scale of genetic distance is provided at the top of the dendrogram.

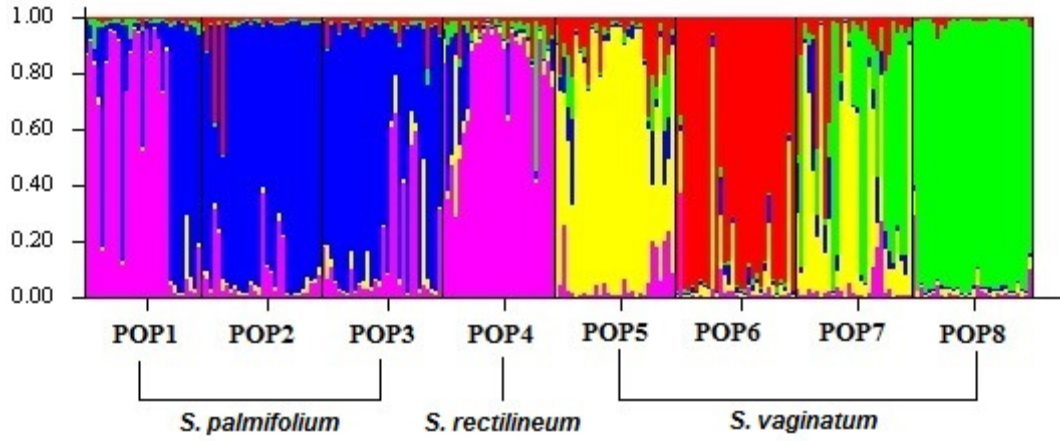


Figure 3. Bayesian admixture proportions (Q) of individual plants of *Sisyrinchium palmifolium* subsp. *palmifolium*, *S. rectilineum* and *S. vaginatum* subsp. *vaginatum* for a K=5 population model. For details of population identification see in Materials and Methods (Table 1).

SUPPLEMENTARY DATA

Table S1. Genetic parameters and summary of F statistics of six SSR loci in eight populations of *Sisyrinchium.* A=number of allele per locus; Ho = observed heterozygosity; He = expected heterozygosity; F_{IT}= overall inbreeding coefficient; F_{ST} = fixation index; F_{IS} = inbreeding coefficient.

Locus	A	He	Ho	F_{IS}	F_{ST}	F_{IT}
VA5	8	0.807	0.609	0.013	0.192	0.202
VA8	9	0.740	0.509	0.078	1.266	0.323
PD3	8	0.721	0.449	0.189	0.267	0.405
PA5	3	0.612	0.533	-0.250	0.329	0.161
L3 (SM-A8)	5	0.617	0.500	-0.076	0.287	0.233
L4 (SM-A10)	6	0.712	0.610	0.026	0.137	0.160
All/Mean	39	0.702	0.535	0.007	0.242	0.247

Departures of within-population inbreeding coefficients (F_{IS}) from HWE are indicated as $p<0.001$.

Table S2. Characterization of microsatellite markers in Rio Grande do Sul populations of *Sisyrinchium palmifolium* subsp. *palmifolium* and *S. vaginatum* subsp. *vaginatum*, including marker source, repeat motif and the following diversity and breeding system parameters calculated at the species level: number of alleles (A), expected (He) and observed (Ho) heterozygosity in each species, within-population inbreeding coefficient F_{IS} and fixation index F_{ST}.

		S. palmifolium subsp. *palmifolium*					*S. vaginatum* subsp. *vaginatum*				
Locus	Repeat motif	A	He	Ho	F_{IS}	F_{ST}	A	He	Ho	F_{IS}	F_{ST}
VA5	(TA)6(GT)7	5	0.695	0.839	-0.221	0.018	8	0.781	0.404	0.302	0.229
VA8	(GT)22(GA)5(GT)4	5	0.511	0.550	-0.080	-0.001	7	0.788	0.445	0.188	0.337
PD3	(CA)7	7	0.717	0.391	0.318	0.301	5	0.674	0.480	0.115	0.256
PA5	(GT)7	2	0.297	0.361	-0.211	-0.004	3	0.565	0.635	-0.262	0.103
L3 (SM-A8)	(TA)2(CA)4TG(CA)3	4	0.689	0.663	-0.004	0.063	2	0.497	0.309	-0.105	0.534
L4 (SM-A10)	(GT)12	4	0.646	0.584	0.085	0.019	6	0.701	0.651	-0.069	0.167
Overall		27	0.593	0.565	-0.024	0.070	31	0.668	0.488	0.038	0.267

Departures of within-population inbreeding coefficients (F_{IS}) from HWE are indicated as $p<0.001$.

Table S3. Results of bottleneck analysis of eight populations accessions of *Sisyrinchium palmifolium* subsp. *palmifolium*, *S. rectilineum* and *S. vaginatum* subsp. *vaginatum* from South of Brazil. Deviations from the mutational equilibrium, $P < 0.05$.

POPULATION	**I.A.M.**				**S.M.M.**			
	Exc. *H* exp	**Def. *H* obs**	**Exc. *H* obs**	***P***	**Exc. *H* exp**	**Def. *H* obs**	**Exc. *H* obs**	***P***
1. Sign test								
POP1	3.31	3	3	0.55528	3.51	3	3	0.48571
POP2	3.13	0	6	0.01905	3.43	1	5	0.19008
POP3	3.22	0	6	0.02304	3.48	3	3	0.49612
POP4	3.43	0	6	0.03412	3.46	5	1	0.05270
POP5	3.13	1	5	0.12855	3.43	4	2	0.21990
PÓP6	3.29	2	4	0.43751	3.53	4	2	0.19526
POP7	3.12	0	6	0.01837	3.43	2	4	0.48661
POP8	3.30	1	5	0.16139	3.43	5	1	0.05479
2. Standardized differences test (T2 values)								
POP1	1.278			0.10056	-0.603			0.27332
POP2	2.114			0.01727	0.944			0.17264
POP3	1.990			0.02330	0.669			0.25178
POP4	1.339			0.09031	-1.040			0.14924
POP5	0.509			0.30551	-0.962			0.16799
POP6	0.737			0.23049	-1.210			0.11313
	2.359			0.00916	1.022			0.15339
POP7								
POP8	0.838			0.20103	-1.245			0.10663
3. Wilcoxon test (probabilities – two tail for *H* excess or deficiency)								
POP1				0.43750				0.68750
POP2				0.01563				0.15625
POP3				0.01563				0.43750
POP4				0.01563				0.43750
POP5				0.15625				0.07813
POP6				0.56250				0.15625
POP7				0.01563				0.15625
POP8				0.10938				0.07813

CAPÍTULO VI

CONSIDERAÇÕES FINAIS

CONSIDERAÇÕES FINAIS

A presente tese está dividida em quatro artigos relacionados a um amplo projeto que visa contribuir para o entendimento de questões relacionadas à evolução e taxonomia da família Iridaceae. Os resultados obtidos nestes trabalhos são inéditos quanto a fatores que contribuíram para a compreensão da evolução, biodiversidade, e relacionamento genético de *Sisyrinchium palmifolium* e espécies relacionadas das seções *Hydastylus* e *Viperella*, presentes nos Campos Sulinos que incluem formações campestres do Bioma Pampa e Mata Atlântica, no sul do Brasil.

O **capítulo II** da tese inclui o primeiro trabalho de caracterização molecular da espécie *Sisyrinchium palmifolium* subsp. *palmifolium,* o qual forneceu um melhor entendimento da variabilidade genética dentro e entre as populações desta espécie. Neste trabalho foi possível verificar o alto grau de polimorfismo desta espécie. A grande variabilidade genética observada principalmente entre os indivíduos dentro das populações garante a esta espécie uma maior adaptação a mudanças ambientais e consequentemente uma maior sobrevivência a longo prazo da espécie. Esta grande variabilidade genética intraespecifica observada pode ser devido à natureza perene destas plantas, o que tem garantido um comportamento como plantas de reprodução cruzada, facilitado pela longa vida dos indivíduos e sobreposição de gerações. A falta de correlação entre distâncias geográficas e genéticas observada para *Sisyrinchium palmifolium* pode ser indica por uma histórica expansão desta espécie no sul do Brasil, como sugerido por Chauveau *et al.* (2011), e pela fragmentação dos habitats, o que tem sido evidenciado nos últimos anos para os Biomas do sul do Brasil. O baixo fluxo gênico observado entre as

populações de *S. palmifolium* subsp. *palmifolium* pode estar relacionado ao movimento do pólen e comportamento do polinizador, a sua distribuição geográfica, e consequente isolamento em ambientes de altas altitudes e ruderal, e a distância entre as populações.

No **capítulo III** as relações de parentesco de *S. palmifolium* com outras espécies da seção *Hydastylus* e *Viperella* presentes na região sul da América do Sul foram evidenciadas. Neste trabalho foi possível verificar que estas espécies pertencentes às duas seções correspondem a um único grupo plantas. Isto é provavelmente devido à recente radiação adaptativa destas espécies e aos eventos de hibridação e fluxo gênico que tem ocorrido entre elas via introgressão. Entretanto, foi verificado que os principais agrupamentos ocorreram entre espécies pertencentes à mesma seção. *S. palmifolium* apresentou amplo polimorfismo com os seus acessos mostrando-se relacionados preferencialmente a outras espécies da seção *Hydastylus* não formando um único agrupamento. Por outro lado, os acessos da espécie *S. antemeridianum* descrita recentemente por Aita *et al.* (2013) apresentaram-se preferencialmente agrupados. Estas diferenças devem-se ao fato que espécies endêmicas tais como *S. antemeridianum* apresentam um menor nível de variação genética do que as plantas com ampla distribuição como *S. palmifolium,* devido principalmente aos restritos fluxos gênicos entre as populações de limitada distribuição geográfica e consequentemente aumento dos efeitos de seleção direcional tais como endocruzamentos, gargalho de garrafa (bottlenecks) e deriva genética. A hipótese sobre a origem das espécies poliploides (*S. marchio*, *S. marchioides* and *S. weirii*) foi levantada pela primeira vez neste estudo, as quais se mostraram relacionadas às espécies diploides de

ambas as seções sugerindo que estas espécies foram originadas através de eventos independentes de aloploidização seguido por evolução divergente resultando nos processos de especiação destas espécies. Além disso, este estudo contribuiu com a identificação de apomorfias e compartilhamento destas entre espécies relacionadas que poderão ajudar na identificação de espécies e a esclarecer as relações filogenéticas entre as espécies da seção *Hydastylus* e *Viperella*. Estas evidências poderão contribuir como base para futuros estudos de DNA barcode para este grupo de plantas.

Nove “primers” de microssatélites foram isolados recentemente para a espécie *Sisyrinchium micranthum* (Tacuatiá et al., 2012b). Com o objetivo de aumentar o número de *loci* disponíveis para o estudo de genética de populações e aumentar a especificidade de detecção de alelos espécie-específico para as espécies de *Sisyrinchium,* outros nove “primers” foram isolados e caracterizados para as espécies *S. palmifolum* (diploide – seção *Hydastylus*) e *S. marchioides* (tetraploide – seção *Viperella*) (**capítulo IV**). Além do isolamento e caracterização de loci de microssatélites, também foram realizados testes de amplificação heteróloga em outras 25 espécies de Iridaceae, sendo 19 espécies de *Sisyrinchium* (incluindo *S. palmifolium* e *S. marchioides*), três espécies de *Calydorea* e três espécies de *Herbertia.* Os resultados mostraram que os “primers” desenvolvidos poderão ser úteis para estudos de outras espécies de Iridaceae, principalmente os “primers” desenvolvidos para *S. marchioides*. Estes loci de microssatélites poderão contribuir para a investigação da origem dos poliploides, identificação de híbridos, para a relação de parentesco entre espécies das seções *Viperella* e *Hydastylus* e detecção de variabilidade genética deste grupo de plantas em

estudos futuros, assim como ajudar a elucidar relações filogenéticas, onde os dados de sequências não forneceram alta resolução e suporte suficiente. No contexto desta tese os loci de microssatélites foram desenvolvidos principalmente para contribuir para o conhecimento dos padrões de diversidade e fluxo gênico entre as principais espécies representantes das seções *Hydastylus* e *Viperella* que são *S. palmifolium* e *S. vaginatum* respectivamente.

Portanto, no **capítulo V**, foi realizado um estudo com 236 indivíduos representantes de oito populações (três populações de *S. palmifolium* subsp. *palmifolium*, quatro populações de *S. vaginatum* subsp. *vaginatum* e uma população de *S. rectilineum*) presentes no estado do Rio Grande do Sul. Os 18 "primers" de microssatélites existentes para *Sisyrinchium* foram testados para estas espécies, entretanto somente seis loci se mostraram polimórficos para estas populações. Apesar do baixo número de loci polimórficos obtidos para análise, os resultados foram satisfatórios e inéditos para este grupo de plantas. Os resultados apontam alto grau de polimorfismo, assim como alta variabilidade genética. Sendo esta variação maior entre indivíduos dentro das populações do que entre as populações. Este padrão também foi observado no **capítulo II**, para as populações da espécie *S. palmifolium* subsp. *palmifolium* utilizando os marcadores ISSR, reforçando a hipótese de fecundação cruzada para esse grupo de plantas. As populações de *S. vaginatum* subsp. *vaginatum* se mostraram mais estruturadas do que as populações de *S. palmifolium* subsp. *palmifolium*. Neste trabalho foi possível reforçar a hipótese de evolução reticulada como no **capítulo III**, com eventos de introgressão ocorrendo recentemente entre estas espécies. Isto foi evidenciado no capítulo V pela falta de correlação entre distância genética e geográfica como verificado utilizando

os ISSRs para as populações de *S. palmifolium* subsp. *palmifolium* (**capítulo II**), e pelo baixo fluxo gênico observado entre as populações de *S. palmifolium* subsp. *palmifolium* e *S. vaginatum* subsp. *vaginatum*, principalmente entre as populações simpátricas coletadas no Morro Santana em Porto Alegre. Os dados de microssatélites forneceram resultados de relacionamento genético entre as espécies congruentes com a delimitação taxonômica estabelecida por Ravenna (2002). As populações de *S. palmifolium* subsp. *palmifolium* agruparam-se e *S. rectilineum* mostrou-se mais relacionado a este grupo do que ao grupo formado com as populações de *S. vaginatum* subsp. *vaginatum*. Este relacionamento preferencial entre as espécies da mesma seção também foi evidenciado no **capítulo III** de análise filogenética. A forte estruturação genética observada entre as populações das três espécies, resultados obtidos a partir dos dados dos marcadores SSRs, principalmente entre populações de *S. vaginatum* subsp. *vaginatum*, sugere a necessidade da implantação de estratégias de conservação *in situ* ou *ex situ* para estas espécies. Portanto, apesar da introgressão ter contribuído para a grande biodiversidade destas espécies, um programa de conservação deverá ser implantado nos biomas sulinos para manter o acesso ao pool gênico destas espécies, ainda mais que estes biomas vem sofrendo grande impacto nos últimos anos devido à forte expansão das atividades agrícolas, assim com pelo decréscimo na vegetação natural do Bioma Mata Atlântica pela expansão da urbanização nesta área o que tem ameaçado as espécies de *Sisyrinchium* pela fragmentação e perda do habitat.

O presente estudo contribuiu com informações inéditas sobre variabilidade genética, evolução, origem de poliploides e estruturação

populacional das espécies das seções *Hydastylus* e *Viperella* na região sul do Brasil. Com base no exposto, *S. palmifolium* e *S. vaginatum* são potenciais espécies modelo para estudos referentes à evolução reticulada, origem de poliplóides. Fica clara a necessidade de uma revisão taxonômica para este grupo de plantas utilizando como base os resultados obtidos nos trabalhos desenvolvidos nesta tese. Para complementar os resultados obtidos até o momento referente a este grupo de plantas, sugere-se: explorar sequências nucleares de cópia única para aumentar o suporte e a resolução dos grupos ainda mal definidos; fazer um estudo filogeográfico destas espécies com um maior número de espécies representantes das seções *Hydastylus* e *Viperella* para obter uma visão mais ampla da estrutura populacional deste grupo de plantas.

CAPÍTULO VII

REFERÊNCIAS BIOBLIOGRÁFICAS DOS CAPÍTULOS I E VI

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