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VARIABILIDADE GENÉTICA E BIOLOGIA DE *SISYRINCHIUM MICRANTHUM* CAV. (IRIDACEAE)

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em todos os momentos

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LISTA DE ABREVIATURAS

AFLP – Amplified Fragment Length Polymorphism

ANCOVA – “Analysis of Covariance”

AMOVA – “Analysis of Molecular Variance”

CTAB – “Hexadecyltrimetyl ammonium bromide”

PEI – Parque Estadual de Itapuã

DMSO – Dimetilsulfóxido

DNA – “Deoxyribonucleic acid”

ICN – Instituto de Ciências Naturais

ISSR – “Inter Simple Sequence Repeats”

LSD – “Least Significant Difference”

NJ – “Neighboor Joining”

PCR – “Polymerase Chain Reaction”

RAPD – “Random Amplified Polymorphic DNA”

RS – Rio Grande do Sul

SSR – “Simple Sequence Repeats”

TFPGA – “Tools for Population Genetic Analyses”

UPGMA – “Unweighted Pair-Group Method Arithmetic Average”

RESUMO

Sisyrinchium micranthum Cav. pertence à família Iridaceae, subfamília Iridoideae, tribo Sisyrinchieae. É uma planta anual com representantes distribuídos no continente americano. No Rio Grande do Sul, observações a campo têm permitido verificar a existência de uma grande variação no porte das plantas, e na coloração das flores, sendo possível encontrar plantas de tamanho *pequeno*, *médio* e *grande*, e coloração violeta, rosa e amarela. Os números cromossômicos descritos na literatura para *S. micranthum* são de 4*x* = 32 e 6*x* = 48, relativos a espécimes coletados no Hemisfério Norte, sendo o provável número cromossômico básico *x* = 8. Não existem estudos abordando aspectos citogenéticos, como comportamento meiótico e fertilidade polínica, e moleculares para esta espécie. Considerando a escassez de estudos sobre a biologia e diversidade genética de *Sisyrinchium micranthum* e sua importância como espécie nativa, este trabalho procurou aliar técnicas de citogenética e marcadores do tipo ISSR-PCR para caracterizar as populações no Parque Estadual de Itapuã e os morfotipos presentes no Rio Grande do Sul. Foi possível verificar que as populações no Parque Estadual de Itapuã (PEI) apresentam-se bastante estruturadas com pouco fluxo gênico entre as mesmas, sendo que a maior parte da variação existente encontra-se intrapopulacionalmente. Foi registrada a ocorrência de indivíduos tetraplóides e hexaplóides no nosso Estado, tendo sido descrita pela primeira vez a presença de populações diplóides. O tipo morfológico *médio* é o mais freqüente, sendo

este, geralmente, de número cromossômico diplóide (*x* = 8). A maioria das plantas examinadas mostrou comportamento meiótico regular e todos os tipos morfológicos apresentaram altas estimativas de índice meiótico e viabilidade de pólen. Os dados citogenéticos e moleculares indicam que *S. micranthum* apresenta populações com mais de um nível de ploidia, evidenciando ampla variação dos caracteres morfológicos, o que dificulta a identificação e classificação das plantas. Foi possível perceber a forte influência da poliploidização na diversificação desta espécie, no entanto, não foi identificado se a mesma ocorre via alo- ou autopoliploidia. Estes dados citogenéticos, moleculares e morfológicos são inéditos e mostram a importância da manutenção destas populações no sul do Brasil, a fim de conservar a variabilidade e possibilitar a continuidade no processo evolutivo da espécie.

ABSTRACT

Sisyrinchium micranthum Cav. belongs to the family Iridaceae, subfamily Iridoideae, tribe Sisyrinchieae. It is an annual plant with representatives distributed on the American continent. In Rio Grande do Sul, field observations have allowed to verify the existence of a great variation in the size of the plants and the color of the flowers. It is possible to find *short, medium* and *tall* plants, and violet, pink and yellow flower colors. The chromosome numbers described in the literature for *S*. *micranthum* are 4*x* = 32 and 6*x* = 48, involving specimens collected in the Northern Hemisphere, and the probable basic chromosome number is *x* = 8. There are no studies looking at cytogenetics, such as meiotic behavior and pollen fertility, and molecular aspects, for this species. Considering the scarcity of studies on biology and genetic diversity of *S. micranthum* and its importance as a native species, this study tried to bring together cytogenetic techniques and markers of the ISSR-PCR type to characterize the populations in the Itapua State Park (Parque Estadual de Itapuã - PEI), and the morphological types present in Rio Grande do Sul state. It could be seen that the populations in the Itapua State Park are quite structured, with little gene flow among the populations, and most of the existing variation is intrapopulational. The occurrence of tetraploid and hexaploid individuals was recorded in our State, and the presence of diploid populations was described for the first time. The *medium* morphological type is most frequent, and it is generally diploid (*x* = 8). Most of the plants examined showed regular meiotic

behavior and all morphological types presented high estimates of meiotic index and pollen viability. The cytogenetic and molecular data indicate that *S. micranthum* presents populations with more than one level of ploidy, showing the wide variation of the morphological characters, which makes it difficult to identify and classify the plants. The strong influence of polyploidization on the diversification of this species could be perceived, but it was not identified whether it occurs by allo- or autopolyploidy. These cytogenetic, molecular and morphological data are new and show the importance of maintaining these populations in the south of Brazil in order to preserve variability and enable continuity in the evolutionary process of the species.

INTRODUÇÃO

FAMÍLIA IRIDACEAE

A família Iridaceae pertence à ordem Asparagales (APGII 2003) e é uma família relativamente grande dentre as plantas monocotiledôneas, com cerca de 1900 espécies e 65 gêneros (Goldblatt and Manning, 2006). Possui ampla distribuição mundial concentrada, principalmente, no Hemisfério Sul, sendo a África o maior centro de diversidade (Goldblatt, 1990).

Esta família é subdividida em quatro subfamílias: Isophysioideae, Nivenioideae, Iridoideae e Crocoideae (Ixioideae). Iridoideae compreende as tribos Trimezieae, Tigridieae, Irideae, Diplarrheneae e Sisyrinchieae, enquanto que a subfamília Crocoideae compreende as tribos Croceae, Watsonieae e Ixieae (Tillich, 2003).

Estudos envolvendo caracteres moleculares evidenciaram que a tribo Sisyrinchieae (Reeves *et al*., 2001), os gêneros *Iris* L. (Souza-Chies *et al*., 1997) e *Moraea* Miller (Goldblatt *et al*., 2002), e a subfamília Nivenioideae (Souza-Chies *et al*., 1997; Reeves *et al*., 2001) não são monofiléticos.

Espécies representantes da família Iridaceae ocorrem preferencialmente em ambientes abertos, como campos, baixadas úmidas e áreas ruderais. São ervas perenes, às vezes anuais, sendo freqüentes os gêneros bulbosos. No aspecto vegetativo, apresentam folhas basais em número variado, cilíndricas ou planas, lineares ou ensiformes, muitas vezes inconspícuas na cobertura

herbácea. As espécies de Iridaceae se caracterizam por suas flores efêmeras, embora de extrema beleza.

No Brasil, encontram-se espécies nativas e exóticas da família Iridaceae. De forma geral, a família está representada por 14 gêneros e 110 espécies (Innes, 1985). Os gêneros mais estudados encontram-se na região sudeste, destacando-se *Neomarica* Sprague, *Trimezia* Salisb. ex Herb. e *Pseudotrimezia* R.C. Foster (Chukr, 1992a, 1992b; Chukr and Giulietti, 2001). No Estado de São Paulo, foi citada a ocorrência de espécies pertencentes a sete gêneros nativos (*Alophia* Herb., *Calydorea* Herb., *Cipura* Aubl., *Eleutherine* Herb., *Neomarica*, *Sisyrinchium* L. e *Trimezia*), além de *Crocosmia* X *crocosmiiflora* (Lemoine) N.E. Br., originária da África e bem aclimatada no Brasil (Chukr and Capellari Jr, 2003).

No Rio Grande do Sul (RS), as espécies nativas destacam-se no período primaveril, quando ocorre o florescimento e é possível percebê-las nos campos naturais. Algumas espécies são consideradas endêmicas e acredita-se que outras ainda não sejam conhecidas. Muitas espécies apresentam elevado risco de desaparecimento em função da ação antrópica sobre o ambiente natural, sendo que as de caráter endêmico são ainda mais suscetíveis a este fator de ameaça.

O Estado apresenta dez gêneros nativos, sendo que o número total de espécies ainda não é conhecido. Estudos florísticos realizados em algumas regiões, e as floras de países limítrofes permitiram a revisão das citações de espécies de Iridaceae para o RS.

O número de espécies de Iridaceae citadas para o Estado até o momento é 43. Destas, cinco espécies são de *Calydorea*, cinco de *Cypella* Herb., duas de *Gelasine* Herb., cinco de *Herbertia* Sweet, uma de *Kelissa* Ravenna, duas de *Neomarica*, uma de *Onira* Ravenna, cerca de 20 espécies de *Sisyrinchium*, uma de *Sympa* Ravenna e uma de *Trimezia*. Os gêneros *Kelissa*, *Onira* e *Sympa* são monoespecíficos criados recentemente (Ravenna, 1981a, 1983).

O GÊNERO *SISYRINCHIUM* L.

Sisyrinchium pertence à subfamília Iridoideae, tribo Sisyrinchieae (Goldblatt *et al.*, 1998). É um gênero com cerca de 200 espécies situadas, principalmente, nas Américas, sendo que algumas têm sido introduzidas ou dispersas em outras partes do mundo (Rudall *et al*., 1986). Dentre as classificações propostas para o gênero, a de Bentham and Hooker (1883) é a mais conhecida, no qual os autores definiram quatro seções para o gênero: *Bermudiana*, *Echthronema, Eriphilema* e *Nuno*. Esta classificação tem sido questionada por alguns autores devido ao posicionamento de alguns gêneros entre as seções propostas.

Esse gênero compreende espécies de pequeno a médio porte, perenes ou anuais. As raízes são geralmente densas e grossas, na forma de tubérculos, ou fibrosas. As folhas são lanceoladas a lineares ou do tipo terete. As flores apresentam cores variadas como creme, amarelo, violáceo e também azul com o centro amarelo e possuem tépalas livres (Goldblatt *et al*., 1998; Chukr and Capellari Jr, 2003).

Johnston (1938) enfatizou a América do Sul como o centro de origem e distribuição de *Sisyrinchium*. Este gênero tem sido enfoque de uma série de trabalhos de Ravenna (1968, 1981b, 2001, 2002a, 2002b), no entanto, tais estudos são pouco esclarecedores, tendo em vista a falta de ilustrações e/ou chaves dicotômicas que contribuam para a determinação das espécies.

A taxonomia de *Sisyrinchium* é notoriamente confusa (Cholewa and Henderson, 1984; Kenton *et al*., 1986; Rudall *et al.*, 1986). Isto se deve especialmente à natureza herbácea e autofértil da maioria de suas espécies, o que causa considerável variação entre, e uniformidade dentro, das populações. Estudos realizados com este gênero reconhecem que o mesmo é constituído de membros com ampla variabilidade (Henderson, 1976). Alguns dos caracteres usados pelos botânicos para delimitar as espécies em *Sisyrinchium* são extremamente variáveis e geralmente de natureza quantitativa (altura da planta), enquanto outros são praticamente invariáveis (textura da superfície do pólen) (Cholewa and 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.

A razão para tamanha confusão quanto à taxonomia de *Sisyrinchium* pode estar relacionada a duas situações: 1) a fragilidade das flores torna difícil a preservação do material em exsicatas, o que conseqüentemente, dificulta a identificação do material seco, além de mascarar a variação floral natural; 2) a variação intrapopulacional de diversas características externas é em alguns

casos extrema e, ao menos que uma grande amostra de indivíduos seja usada como base para a avaliação do caráter, a identificação pode ser difícil ou até impossível (Henderson, 1976).

A grande variação morfológica encontrada neste gênero parece ainda refletir uma influência ambiental. Fatores como o pH do solo e estresse hídrico, possivelmente têm um papel importante em diversas características externas das plantas (Ingram, 1968; Henderson, 1976). Dados morfológicos, citológicos, genéticos e geográficos são fundamentais para dar suporte à caracterização taxonômica.

SISYRINCHIUM MICRANTHUM CAV.

Sisyrinchium micranthum Cav. (Fig. 1) se caracteriza por folhas e escapos florais planos, com inflorescências terminais com flores de ampla variação de coloração, incluindo flores brancas, amarelas, lilases e com matizes variegados (Chukr and Capellari Jr., 2003). Nestas plantas, os filetes são unidos cerca de 1/2 a 2/3 de sua extensão, com grande densidade de elaióforos (glândulas florais especiais que produzem óleos lipídicos, utilizados por alguns polinizadores) na base. A espécie ocorre entre México e Argentina, além de ocorrer de forma naturalizada na Austrália, Malásia e nas Ilhas Fiji (Innes, 1985). No Brasil, habita diferentes ambientes, como campos, matas ou locais antropizados, sendo citada para os estados do Rio Grande do Sul, Santa Catarina, Paraná, São Paulo e Rio de Janeiro (Chukr and Capellari Jr., 2003). É uma espécie herbácea, normalmente citada como anual (Johnston, 1938; Innes, 1985, Goldblatt, 2003).

Sisyrinchium micranthum apresenta uma grande variação no porte dos indivíduos (Chukr and Capellari Jr., 2003). No Rio Grande do Sul (RS), Brasil, também se tem observado uma grande variação, sendo reconhecidas a campo, plantas de tamanho *pequeno*, *médio* e *grande.* A variação quanto à coloração das flores é também evidente havendo indivíduos de coloração violeta (a mais freqüente), rosa e amarela. Estudos vêm sendo realizados a fim de estabelecer categorias morfológicas nas plantas de *S. micranthum* ocorrentes no Sul do Brasil.

Esta espécie é conhecida popularmente por “Scourweed” (HEAR) ou “espadilla”, sendo descrita como erva de uso medicinal (Caro, 2004).

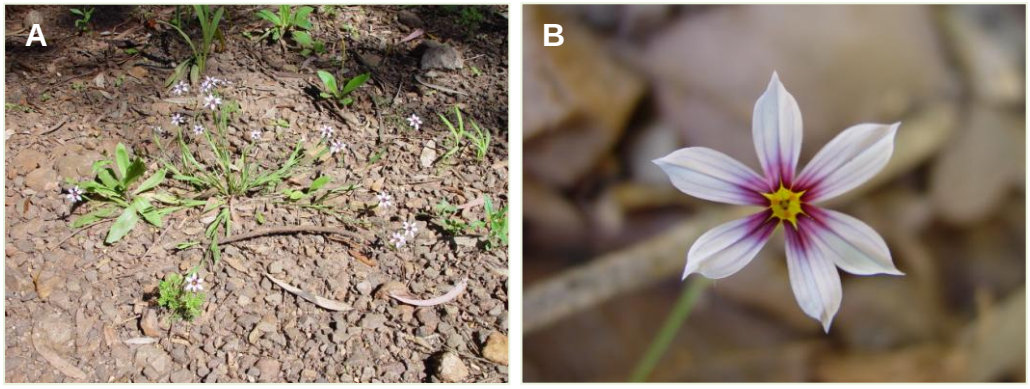

Fig. 1. *Sisyrinchium micranthum* Cav. (Iridaceae). A – Planta; B – Flor. Fotos cedidas por Lilian Eggers.

BIOLOGIA REPRODUTIVA

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

ou não a formação de gametas e o segundo define os padrões pelos quais os gametas são reunidos (Bodanese-Zanettini and Cavalli, 2003).

Espécies vegetais podem apresentar exclusivamente um dos modos de reprodução, assexual ou sexual, e um sistema de cruzamento, autogamia ou alogamia, mas é comum a prevalência de um modo de reprodução e de um sistema de cruzamento com eventos esporádicos do outro tipo (Bodanese-Zanettini and Cavalli, 2003). No entanto, a transição evolutiva de fecundação cruzada para autofecundação tem ocorrido em muitos grupos de plantas, de forma que diferentes sistemas de cruzamento têm sido observados tanto entre espécies relacionadas, quanto entre populações distintas da mesma espécie (Jain, 1976). Esta mudança está, geralmente, associada com mudanças na biologia floral, história de vida da planta e ecologia (Barret *et al.,* 1996; Ornduff, 1969 *apud* Goodwillie, 2005).

Com relação à biologia floral, nectários septais e perigoniais (estames e tépalas) raramente ocorrem juntos na mesma flor em monocotiledôneas (Smets *et al.,* 2000 *apud* Rudall *et al.,* 2003), mas na família Iridaceae ambos os tipos ocorrem, sendo que algumas espécies também apresentam células epidermais produtoras de óleos e elaióforos concomitantemente (Rudall *et al.,* 2003).

Flores que oferecem óleos como recompensa aos insetos foram registradas, até o momento, em apenas oito famílias de plantas (Machado, 2004), estando a família Iridaceae entre as mais importantes. Na região Neotropical, flores produtoras de óleo são visitadas, principalmente, por grupos de abelhas especializadas pertencentes às tribos Centridini, Tapinotaspidini e

Tetrapediini (Apidae, Apinae) (Buchmann, 1987). Em relação às espécies de *Sisyrinchium* com flores produtoras de óleo, Cocucci and Vogel (2001) mencionam a existência de especializações e adaptações mútuas antes não conhecidas, relatando a ocorrência de estreitas associações entre as flores e espécies de abelhas dos gêneros de Tapinotaspidini.

SISYRINCHIUM MICRANTHUM

A maioria das espécies de *Sisyrinchium* não possui elaióforos, como por exemplo, *Sisyrinchium palmifolium* L. e *S. vaginatum* Spreng. (Cocucci and Vogel, 2001; Freitas and Sazima, 2003). Truylio *et al*. (2002) descreveram a biologia floral e a polinização de *S. micranthum* em São Francisco de Paula, RS, e verificaram que esta espécie floresce no período da manhã. Dentre as espécies presentes no Estado que já foram vistas ocorrendo próximas à *S. micranthum*, *S. palmifolium* inicia a abertura das flores às 15:30 h (Cocucci and Vogel, 2001) enquanto que as flores de *S. vaginatum*, no sudeste do Brasil, abrem entre 13:00 e 15:00 h (Freitas and Sazima, 2003).

As flores de *Sisyrinchium micranthum* são protogínicas, pois a receptividade do estigma começa seis a sete horas antes do amadurecimento das anteras (Truylio *et al,* 2002). Além disso, experimentos de polinização controlada indicaram que *S. micranthum* é auto-incompatível. No entanto, a protandria (disponibilidade do pólen antes que o estigma esteja receptivo) parece ser muito mais freqüente dentro do gênero *Sisyrinchium* (Henderson, 1976; Cocucci and Vogel, 2001). A dicogamia é um mecanismo que geralmente facilita a fecundação cruzada, visto que o amadurecimento do pistilo e dos

estames ocorre em épocas distintas, dificultando a autofecundação. No entanto, Henderson (1976) estudou espécies de *Sisyrinchium* no Hemisfério Norte e verificou que conforme o nível de ploidia, o grau de expressão da protandria mostrava-se alterado, tendo efeitos diretos no sistema de cruzamento. Neste mesmo trabalho, através de experimentos de fecundação manual foi possível verificar que as espécies tetraplóides eram auto-incompatíveis, enquanto espécies com nível de ploidia superior eram autoférteis. Além disso, observações no periodo de abertura floral detectaram que a protandria, frequentemente presente, promovia a fecundação cruzada, mesmo em espécies autocompatíveis. Contudo, foi observado que quanto maior fosse o nível de ploidia das espécies investigadas, menos pronunciado era o intervalo de tempo entre a maturação das anteras e do estigma. Desta forma, enquanto as espécies tetraplóides eram protândricas, a maioria dos octoplóides e dodecaplóides apresentava um intervalo de maturação muito curto entre as partes reprodutivas ou praticamente ausente, facilitando a autofecundação nas espécies autocompatíveis. Em *Sisyrinchium bermudiana* L. as flores são protândricas, mas também já foi descrita uma variação no comprimento do filete. Conseqüentemente, quando a antera apresenta-se no mesmo nível do estilete, existe uma grande probabilidade de ocorrer a autopolinização. Esta espécie ocorre em uma ampla área da América do Norte, é autocompatível e forma uma série poliplóide com 2*n* = 32, 64 e 96 (Kenton *et al.*, 1986; Ingram, 1968), no entanto, para esta espécie não foi determinado se existe relação entre nível de ploidia e autopolinização (Ingram, 1968).

A respeito da polinização, a maioria das espécies africanas de Iridaceae é polinizada por abelhas ou outras espécies de Hymenoptera, enquanto o grupo de plantas restante é polinizado por insetos das ordens Coleoptera, Diptera, e Lepidoptera, ou por pássaros da família Nectarinidae. Em muitas espécies, os atrativos para os polinizadores são a pigmentação do perianto complementada por odores florais, mas a forma das flores e a orientação das tépalas numa simetria floral funcional particular também são importantes para alguns polinizadores (Golblatt and Manning, 2006).

Os polinizadores de *Sisyrinchium micranthum* são abelhas coletoras de óleo da família Apidae, tribo Tapinotaspidini (Cocucci and Vogel, 2001; Truylio *et al.*, 2002). Contudo, sirfídeos e pequenas abelhas coletoras de pólen já foram vistas visitando as flores de *S. micranthum* (Truylio *et al.*, 2002; Freitas and Sazima, 2006). Para as espécies de *Sisyrinchium* da América do Norte já foram observados como polinizadores abelhas solitárias da família Megachilidae (Henderson, 1976) e Halictidae (Cholewa and Henderson, 1984), assim como para *Sisyrinchium palmifolium* na Argentina (Cocucci and Vogel, 2001). Polinização via pequenas abelhas coletoras de pólen foi registrada para *S. vaginatum* durante o verão numa região de cerrado de Minas Gerais (Barbosa, 1997), enquanto Freitas and Sazima (2003) observaram durante o inverno na Serra da Bocaína, Rio de Janeiro, que sirfídeos, ao invés de abelhas, foram os principais polinizadores. Isto pode estar relacionado com o fato de que na área estudada, o pico de florescimento ocorre no inverno, enquanto em outras áreas ocorre no verão. Sendo que no inverno ocorre decréscimo na diversidade e no número de abelhas.

ESTUDOS CITOLÓGICOS

CITOGENÉTICA DA FAMÍLIA IRIDACEAE

Estudos envolvendo citologia cromossômica em Iridaceae têm demonstrado uma variação não usual quanto às características do cariótipo, incluindo o número básico, nível de ploidia, e tamanho e forma dos cromossomos (Goldblatt and Takei, 1997). Portanto, a análise citológica constitui um fator relevante a ser considerado na sistemática e evolução desta família.

Das 1900 espécies que compõem a família, pouco mais de 1000 já têm seu número cromossômico determinado, sendo estas, em sua maioria, espécies do Hemisfério Norte e África (Goldblatt andTakei, 1997). Tais estudos normalmente se limitam à contagem cromossômica. Análises cariotípicas, de comportamento meiótico, de avaliação polínica e de tamanho genômico são ainda escassas. A maior parte dos trabalhos relativos à citogenética, taxonomia e evolução de Iridaceae é do grupo do Dr. Peter Goldblatt, do Missouri Botanical Garden, o qual vem trabalhando com esta família desde a década de 1960.

Números cromossômicos básicos foram sugeridos para todas as subfamílias e tribos com base em algumas das espécies já reconhecidas (Goldblatt and Takei, 1997). A maior parte dos números básicos ancestrais é relativamente alta em Iridaceae, o que sugere que a poliploidia tenha irrompido cedo na evolução da família, tendo assim uma origem paleopoliplóide.

Segundo Goldblatt and Takei (1997), a neopoliploidia (relativa à poliploidia intragenérica) é comum nos membros da família ocorrentes no Hemisfério Norte, sendo estimada em torno de 60%. Cerca de 5% das espécies de Ixioideae e 10% de Iridoideae são poliplóides e 10% das demais espécies apresentam tanto populações diplóides como poliplóides. A poliploidia parece ter sido mais importante na evolução das Iridáceas das Américas do Sul e Central, onde, por exemplo, praticamente todas as espécies já investigadas da tribo Tigridieae são tetra e hexaplóides. Assim, a poliploidia foi um fator evidentemente importante na diversificação de Iridaceae, e muitos gêneros têm números básicos superiores a $x = 10$.

O número básico para a família é ainda incerto. O provável número básico para as subfamílias Nivenioideae, Iridoideae e Crocoideae (Ixioideae) é $x = 10$, a julgar pela distribuição dos números básicos dentro dos gêneros menos especializados (Goldblatt, 1990). Na verdade, uma imensa variação quanto ao número básico é verificada dentro de cada tribo, e mesmo gênero. Por exemplo, dentro de Crocoideae são encontradas espécies com "*x*" variando desde três até 16 cromossomos. Goldblatt and Takei (1997) sugerem que, tal variação, freqüente dentro de alguns gêneros, é resultado de disploidia reducional (ou decrescente), evidenciada por translocações recíprocas desiguais. A disploidia é uma alteração no cariótipo decorrente de perda ou ganho de um ou poucos cromossomos, que são geralmente originados a partir de alterações na meiose de um dos parentais (Heywood, 1970; Stebbins, 1971).

A ocorrência de populações aneuplóides para algumas espécies poliplóides dentro da família é relatada nos trabalhos de Sharma and Talukdar (1960), Ingram (1968) e Goldblatt (1982), o que gera variação adicional quanto às contagens cromossômicas.

Dentro da subfamília Iridoideae foi verificada uma ampla diversidade quanto à morfologia e tamanho cromossômico. Os cromossomos podem variar desde muito pequenos, em alguns gêneros da Austrália e América do Sul, até muito grandes, particularmente no Velho Mundo (Goldblatt *et al.* 1984). Existem gêneros, como *Libertia* Spreng. e *Orthrosanthus* Sweet, cujos cromossomos são pequenos, apresentando cerca de 1 µm. Já no subgênero *Grandiflora*, os cromossomos têm de 10 a 14 µm de comprimento (Goldblatt and Takei, 1997).

A bimodalidade (presença de dois grupos distintos de cromossomos) é comum em Iridoideae (Goldblatt and Takei, 1997). Molseed (1970) descreve para *Tigridia* Juss. um cariótipo bimodal, tendo dois pares de cromossomos grandes e 12 pequenos. Em *Orthrosanthus chimboracensis* (Kunth) Baker, Kenton and Heywood (1984) verificaram a presença de bimodalidade, havendo cromossomos maiores, com 2 - 2,3 µm e menores, com 0,5 - 1,2 µm.

Goldbaltt *et al.* (1984) realizaram a medida do conteúdo de DNA em 30 espécies de 19 gêneros de Iridaceae (subfamílias Crocoideae e Iridoideae). Os autores sugerem que aumento e redução no tamanho do genoma ocorreram durante a evolução do grupo. Valores entre 1,2 pg e 4,9 pg de DNA foram encontrados para sete espécies de diferentes gêneros de Ixioideae. Tais resultados são consistentes com as observações cariotípicas para esta

subfamília, na qual cromossomos pequenos são característicos e sem variação substancial na quantidade total de material cromossômico. Já o genoma diplóide de Iridoideae variou grandemente, com extremos entre 6,4 pg e 65,1 pg. As razões para tamanha variação entre os gêneros e espécies de Iridoideae, ainda não estão esclarecidas.

CITOGENÉTICA DO GÊNERO *SISYRINCHIUM* L.

Informações citológicas quanto às espécies de Iridaceae ocorrentes na América do Sul são realmente escassas, principalmente para a tribo Sisyrinchieae (subfamília Iridoideae) (Goldblatt, 1982; Kenton *et al*., 1986).

Apesar do gênero *Sisyrinchium* ser o maior desta tribo, são poucos os trabalhos de citogenética existentes, sendo em sua maioria para espécies da América do Norte. Em tais estudos, o gênero foi descrito como tendo cromossomos pequenos, números cromossômicos elevados e altamente variáveis devido aos eventos de poliploidia e hibridação natural (Kenton *et al.*, 1986).

Considerando a classificação de Bentham and Hooker (1883) e os trabalhos citogenéticos desenvolvidos baseando-se na mesma, o número cromossômico básico é $x = 8$ para a seção *Bermudiana* sendo que todas as espécies apresentam cromossomos pequenos. São encontrados desde espécies diplóides ($2n = 16$) até dodecaplóides ($2n = 96$). Os genomas haplóides desta seção contêm entre 0,37 e 0,73 pg de DNA. Portanto, embora muitas espécies apresentem elevado nível de ploidia, o tamanho do genoma

básico é relativamente constante e menor do que a maioria dos diplóides das outras seções.

Segundo Goldblatt (1982) e Rudall *et al.* (1986), o nível de ploidia aumenta com a latitude: diplóides no México e Sul dos EUA, tetraplóides no Norte dos EUA, e aqueles com maiores níveis de ploidia (octa e dodecaplóides) no Canadá. Os altos níveis de ploidia em zonas temperadas do Hemisfério Norte podem indicar uma necessidade de aumento da heterozigosidade nas espécies colonizadoras.

Rudall *et al.* (1986), relataram que dentro da seção *Echthronema* as espécies são variáveis quanto ao número básico, nível de ploidia e tamanho cromossômico, sendo separadas em dois grupos com base no número básico (x = 8 e x = 9) e tamanho dos cromossomos (pequenos e variáveis). A variação no tamanho cromossômico é mais pronunciada entre os diplóides. Não se sabe se as diferenças citológicas estão relacionadas a mudanças morfológicas externas. Contudo, a distinção citogenética pode ser correlacionada com o ciclo de vida e preferência de habitat em algumas espécies.

Apesar dos dados citológicos para a seção *Echthronema* serem limitados a análises de poucas espécies, parece que naquelas com x = 8, assim como na *Bermudiana*, o tamanho genômico é pequeno e constante (0,32 a 0,58 pg), embora o nível de ploidia seja variável (Kenton *et al.*, 1986). Já as espécies com x = 9 exibem a maior variação quanto ao tamanho do genoma na seção *Echthronema*, variando de 0,25 a 2,10 pg. A maioria das espécies deste

grupo é diplóide (2*n* = 18) ou tetraplóide (2*n* = 36), com uma distribuição entre o extremo Sul do Chile e México.

Ainda dentro da seção *Echthronema* são encontrados citotipos com números cromossômicos intermediários (*Sisyrinchium elmeri* Greene*,* 2n = 34*; S. longipes* (E.P. Bicknell) Kearney & Peebles*,* 2*n* = 34 *e S. jamesonii* Baker*,* 2*n* = 68*)* àqueles para as espécies baseadas em *x* = 8 e *x* = 9. A provável origem destes números intermediários é a partir de uma hibridação ancestral, uma vez que híbridos interespecíficos são encontrados na natureza (Shinners, 1962). Estudos meióticos em *Sisyrinchium* são praticamente inexistentes, embora estes possam trazer inúmeras informações relevantes quanto à origem dos poliplóides (por auto ou alopoliploidia). Em populações dodecaplóides de *Sisyrinchium* presentes no extremo oeste do Canadá, foram observadas irregularidades na meiose, como retardatários (quatro ou cinco univalentes) e uma baixa taxa de coloração do pólen. Considerando que tais indivíduos apresentaram características morfológicas relacionadas com *S. idahoense* E.P. Bicknell, *S. montanum* Greene e *S. septentrionale* E.P. Bicknell os autores consideraram a possibilidade de tais espécimes serem híbridos interespecíficos (Henderson, 1976). Cholewa and Henderson (1984) encontraram indivíduos dodecaplóides semelhantes a *S. septentrionale* (geralmente *n* = 16) na mesma região estudada por Henderson (extremo noroeste do Canadá) com meiose freqüentemente normal, fato que, segundo os autores, indicava a existência de híbridos estáveis. Segundo Kenton *et al.* (1986) a realização de cruzamentos interespecíficos e a análise meiótica dos híbridos artificiais formados poderiam auxiliar na compreensão das variações no número básico deste grupo.

Os números cromossômicos descritos para *Sisyrinchium micranthum* são de 2*n* = 32 para espécimes introduzidos no Texas (Goldblatt, 1982), e 2*n* = 48, relativos a indivíduos coletados na Colômbia (Kenton and Heywood, 1984) e Nicarágua (Goldblatt and Takei, 1997).

Apesar da importância de análises de comportamento meiótico e viabilidade polínica em estudos citogenéticos que visem à caracterização de uma população ou espécie, em *Sisyrinchium micranthum* não existem investigações com esta abordagem realizadas até o presente momento.

ACESSO À VARIABILIDADE GENÉTICA

O uso de marcadores moleculares, tais como RAPD ("Random Amplified Polymorphic DNA"), SSR ("Simple Sequence Repeats"), AFLP ("Amplified Fragment Length Polymorphism") e ISSR ("Inter Simple Sequence Repeats"), tem sido amplamente difundido na caracterização da variabilidade genética de diferentes famílias. Dentre estas, a técnica de ISSR combina os benefícios das análises por AFLP e SSR com a universalidade do RAPD. Além disto, possui alta reprodutibilidade e segrega, principalmente, como marcador dominante (Reddy *et al*., 2002).

A técnica de ISSR é baseada no método de PCR ("Polymerase Chain Reaction") e envolve a amplificação de um segmento de DNA presente a uma distância passível de amplificação entre duas regiões de microssatélites idênticas e orientadas em direções opostas. São utilizados iniciadores de 16 a 25 pb, constituídos de seqüências di, tri, tetra ou penta-nucleotidicas. Não

necessita do conhecimento prévio das seqüências alvo como na técnica de SSR. Os produtos amplificados possuem entre 200 – 2000 pb e podem ser detectados em gel de agarose ou de poliacrilamida. Possui alta probabilidade de detecção de polimorfismo devido ao fato das taxas evolutivas na região de microssatélites serem altas (Reddy *et al*., 2002).

A aplicabilidade da técnica de ISSR é ampla: “fingerprinting” genômico, mapeamento de genes, determinação da freqüência de motivos SSR, seleção assistida, biologia evolutiva, filogenia e análise da diversidade genética (Reddy *et al*., 2002).

Até agora, poucos estudos têm sido realizados utilizando marcadores moleculares nucleares para investigar a diversidade de espécies de Iridaceae (Wróblevska *et al*., 2003; Caiola *et al*., 2004; Meerow *et al.*, 2005; Meerow *et al.*, 2007).

A análise da variabilidade genética avaliada aos níveis inter e/ou intrapopulacional é de extrema relevância em estudos que visam à caracterização biológica de uma espécie e também sua conservação. Permite relacionar sua condição atual e sua história evolutiva possibilitando delinear propostas de manejo e de manutenção da espécie.

OBJETIVOS

Considerando a escassez de estudos sobre a biologia e diversidade genética de *Sisyrinchium micranthum* e sua importância como espécie nativa, este trabalho tem como objetivo principal caracterizar populações de plantas descritas como *S. micranthum* presentes no Rio Grande do Sul aliando as técnicas de citogenética e biologia molecular. A partir de tais investigações espera-se contribuir para a taxonomia, biologia reprodutiva e evolutiva da espécie.

OBJETIVOS ESPECÍFICOS

- Avaliar a variabilidade intra e interpopulacional de *Sisyrinchium micranthum* através da técnica de ISSR-PCR no Parque Estadual de Itapuã, Viamão, RS;
- Estabelecer uma associação entre o parâmetro morfométrico tamanho das anteras florais e os estágios da microsporogênese, a fim de facilitar análises citogenéticas como determinação do número cromossômico, comportamento meiótico e fertilidade polínica;
- Determinar o número cromossômico da espécie para o Rio Grande do Sul;
- Analisar comparativamente os diferentes tipos morfológicos da espécie, no RS, quanto ao comportamento meiótico, à viabilidade e ao tamanho dos grãos-de-pólen.

GENETIC VARIABILITY OF *SISYRINCHIUM MICRANTHUM* CAV. (IRIDACEAE) IN SOUTHERN BRAZIL

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GENETIC VARIABILITY OF *SISYRINCHIUM MICRANTHUM* CAV. (IRIDACEAE) IN SOUTHERN BRAZIL[1]

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Sisyrinchium micranthum Cav. is a member of the family Iridaceae, distributed over the American continent. In Brazil it is found in depredated areas, coastal regions and is very common in urban centers such as public parks in spring. There are two chromosome counts for North American specimens, 2*n* = 32 and 48, but the chromosome numbers for the Brazilian specimens are unknown. Population analyses with DNA molecular markers have never been performed for this species despite its wide distribution and morphological variation. To study the population genetics and to analyze the chromosome numbers of *S. micranthum* five natural populations were accessed in a conservation park belonging to the Atlantic Forest Biome in southern Brazil. The chromosome counts showed the occurrence of hexaploid individuals with 2*n* = 48 and, for the first time, diploid individuals (2*n* = 16), inside the conservation park. Molecular analyses showed that populations are highly structured with low gene flow among them. The population with 2*n* = 48 was less genetically variable being more distant from the other populations. The molecular and cytogenetic data provided new insights about the genetic diversification and mating system of this species.

Key words: basic chromosome number; Iridaceae; ISSR-PCR; mating system; population genetics; *Sisyrinchium micranthum*

Sisyrinchium micranthum Cav., a member of the family Iridaceae, is a herb species with violet, yellow or pink flowers. This species produces floral oil in trichomatic structures called elaiophores, as a reward to their pollinators (Cocucci and Vogel, 2001; Truylio et al., 2002). Its distribution spans the Americas (Johnston, 1938; Goldblatt, 2003), between South Argentina and Mexico. In Brazil it is found in depredated areas, coastal regions and is very common in urban centers such as public parks in spring. In South Brazil, when this species is observed, there is a remarkable morphological variation in size of the plants and *short*, *medium* and *tall* plants are recognized.

Family Iridaceae is represented by ca. 65 genera and 1900 species (Goldblatt and Manning, 2006). It has a worldwide distribution with a marked concentration on the southern continents and its main center of radiation is in South Africa (Goldblatt, 1990). Some Iridaceae genera have been much studied because of their economic importance as ornamental plants, food or spice. Otherwise, most of the species without economic value is poorly known, such as *Sisyrinchium* L. species. This genus is represented by approximately 200 species in America (Rudall et al., 1986). Data on its biology, cytogenetics and leaf anatomy are available, especially for North American species (Ingram, 1968; Henderson, 1976; Cholewa and Henderson, 1984; Goldblatt et al., 1984; Kenton et al., 1986; Rudall et al., 1986; Goldblatt and Takei, 1997). Basic information involving taxonomy and chromosome number is unknown for almost all species of *Sisyrinchium* L. from South America, above all Brazilian specimens. The same situation occurs considering population genetics using DNA markers,

since there are not available studies for this genus as well as in most Iridaceae species.

Iridaceae species including *Iris* L.*, Moraea* Mill.*, Crocus* L.*, Gladiolus* L. and even genus *Sisyrinchium***,** have extensive polyploid series (Ingram, 1968; Henderson, 1976; Goldblatt, 1982; Cholewa and Henderson, 1984; Kenton and Heywood 1984; Kenton et al., 1986; Rudall et al., 1986; Goldblatt and Takei, 1997). Considering species alone, *S. bermudiana* L. has 2*n* = 32, 64 and 96, and *S. convolutum* Nocca has 2*n* = 36 and 72 (Kenton et al., 1986).

At least half of the natural species of flowering plants are polyploid (Hieter and Griffiths, 1999).The polyploidy has being considered an important phenomenon involved in the origin and evolution of Angiosperm species (Masterson, 1994). The same influence seems to be present in *Sisyrinchium micranthum*, since then there are two different chromosome numbers for this species until now: 2*n* = 32 for introduced specimens collected in Texas (Goldblatt, 1982) and 2*n* = 48 for native individuals collected in Colombia, (Kenton and Heywood, 1984) and Nicaragua (Goldblatt and Takei, 1997).

Besides basic characterization, population genetics analyses are very important in the context of conservation because it supplies subsidies to establish strategies of conservation of the genetic patrimony, helping in studies to characterize the diversity of a region. In Southern Brazil, the Itapua State Park (Parque Estadual de Itapuã - PEI) is a state conservation park which preserves the remaining original vegetation of the region and a great diversity of ecosystems belonging to the Atlantic Forest Biome. The South American Atlantic Forest is one of the highest-priority hotspots on Earth and it has already

lost over 90% of its biodiversity (Fonseca et al., 2004). *Sisyrinchium micranthum* has being observed in the PEI distributed over the park, mainly in ecological paths.

Among several techniques to study genetic variability within and among populations, Inter Simple Sequence Repeats (ISSR) is a powerful molecular technique due to its high capability to detect polymorphisms at various loci. It is based on polymerase chain reaction (PCR) and the advantages are that it does not require prior knowledge of the nucleotide sequence to be analyzed, it is low cost, the laboratory procedures can easily be adapted to other species, and, above all, it does not need large amounts of DNA. The latter characteristic is very important because some populations of *Sisyrinchium micranthum* are composed by tiny individuals. Until now, few studies have been carried out using nuclear DNA markers to investigate genetic diversity of Iridaceae species (Wróblevska et al., 2003; Caiola et al., 2004; Meerow et al., 2005; Meerow et al., 2007).

Regarding the lack of studies about population variability of *Sisyrinchium micranthum* and the importance of characterizing the genic pool of this species in a conservation area, this work aims to analyze *S. micranthum* populations in the PEI using ISSR primers and chromosome counts.

MATERIAL AND METHODS

Population sampling

In 2005, a total of five populations of *Sisyrinchium micranthum* were sampled in the Itapua State Park (50°50’ and 51°05’ W, 30°20’ and 30°27’ S) located in

Viamão, RS, 57 km from Porto Alegre (Fig. 1). The study sites are Praia de Fora, Pedra da Visão, Pedra da Grota and Praia da Pedreira. Four populations with light violet flowers and one population with two colors of flowers, light violet and light yellow, were collected. Table 1 shows the names of populations per site, the coordinates, the number of individuals collected and the color of the flowers. The number of collected individuals agrees with the population size, so that all the individuals with flowers had a leaf sample collected to carry out the molecular analyses. Voucher specimens have been deposited in the herbarium of the Instituto de Biociências, Universidade Federal do Rio Grande do Sul (ICN).

DNA isolation and ISSR-PCR amplification

Total genomic DNA was extracted from 10-50 mg of silica gel-dried leaves using hexadecyltrimetyl-ammonium bromide (CTAB) according to the Doyle and Doyle method (1987) with modifications. A set of twelve ISSR primers was tested and six that generated good patterns with a representative sample group were further used for amplification of plant DNA of all the populations. PCR reactions were carried out in 25 µl volume. The conditions had to be optimized for each primer and after the reactions were performed as follows (depending on the primer): 4% DMSO, 1x buffer, 4.6 – 5.0 mM $MgCl_2$, 0.48 – 0.8 mM of each dNTP (Invitrogen^{TM} Brazil), 0.4 – 0.6 µM of each primer, 1 U *Taq* polymerase (CentBiot$^{@}$/Brazil) and 10 ng of genomic DNA. Amplifications were executed using a Techne TC412 Termocycler with initial denaturation of 5 min at 92°C, followed by 35 cycles of 1 min at 94°C, 1 min at 42 – 45°C (depending

on the primer Ta), 2 min and 30 seconds at 72°C and a 5-min final extension step at 72°C. PCR products were analyzed on 1.5% agarose gels and stained with GelRed™ (Brazil).

Cluster analyses

Bands were scored for presence (1) or absence (0), and a binary file was prepared. An unbiased genetic distance matrix (Nei, 1978) was generated by TFPGA v. 1.3 (Tools for Population Genetic Analyses) (Miller, 1997) to create unweighted pair-group method arithmetic average (UPGMA), computing 1000 permutations and estimating the confidence limits of the dendrogram. One pairwise difference matrix was performed by ARLEQUIN v. 3.11 (Excoffier and Schneider, 2005) with 1000 permutations and used to create UPGMA and neighbor-joining (NJ) dendrograms with MEGA 4.0 (Tamura et al., 2007). The amplifications for population ESC172 resulted in a particular pattern of bands at seven loci, so that all individuals with light violet flowers (ESC172vb) presented a band while all individual with light yellow flowers (ESC172a) did not have a band and vice-versa. But in the other populations (all of them with light violet flowers) there was no similar pattern. Because of this the two colors were treated like subpopulations in the case of ESC172 to carry out the analyses.

Statistical analyses

The number of polymorphic loci, Nei´s genetic identity (1973), and Shannon information index of phenotypic diversity (Shannon and Weaver, 1949) were computed with POPGENE v. 1.32 (Yeh et al., 2000) assuming all loci were

dominant and in Hardy-Weinberg equilibrium. Correlations between the Nei diversity (*h*) and Shannon diversity (*I*) were conducted in SPSS v. 10.0 (SPSS Inc., Chicago, USA). A hierarchical analysis of molecular variance (AMOVA) (Excoffier et al., 1992) was computed with ARLEQUIN v. 3.11 to determine the variance components and their significance levels. So it was conducted considering the subpopulations from population ESC172 one group and the other populations another group.

To test a correlation between the pairwise Φ_{ST} matrix generated by ARLEQUIN and unbiased genetic distance matrix generated by TFPGA, and between genetic and geographic distances (in km) among populations, a Mantel Test was performed using TFPGA, computing 10 000 permutations.

Bayesian genetic structure analysis

STRUCTURE v. 2.2 (Falush et al., 2007) was used in order to obtain additional insights about gene flow and population subdivision, thus it was estimated the most likely number of populations (*k*). The analyses were carried out with the admixture model and independent allele frequencies, with no prior information about the population origin. The program was ran for 100 000 iterations after a burn-in length of 10 000 iterations testing population subdivision from $k = 1$ to $k = 10$. Ten runs were carried out for each *k*. Individual and average admixture proportions (*Q*) for each population in each genetic cluster found by the program were recorded for the model.

Chromosome counts

Floral buds from at least ten individuals per population were collected and fixed in 3:1 ethanol: acetic acid for 12 - 24 hours at room temperature. After fixation, buds were transferred to 70% ethanol and stored in a freezer at -18°C. Pollen cell wall was very stiff making it difficult to acquire a good chromosome spread. Thus, prior to analyses, three anthers present in each flower were washed in distilled water, then hydrolyzed in pectinase-cellulase 2% at 37°C for 30 min. For slide preparation, the anthers were washed again in distilled water and squashed in 1% propionic carmine. Slides were examined and documented with a Zeiss Axioplan Universal photomicroscope. It was not possible to investigate the chromosome numbers using mitotic cells because it was difficult to obtain enough material with good quality and quantity to analyze.

RESULTS

The six primers produced a total of 80 scoreable bands of which 98.75% were polymorphic. The ISSR fragments generated an average of 13.2 bands per primer. The size of amplified products ranged from 325 to 1800 bp (Table 2). The percentage of polymorphic loci varied from 43.75% to 78.75%, the Nei's diversity indices within each population are shown in Table 3. Nei diversity (*h*) and Shannon diversity (*I*) were highly correlated ($r = 1.000$, $P < 0.01$). Population 172 had the lowest values for percentage of polymorphic loci (43.75%), Nei diversity (0.1496) and Shannon index (0.2237) while the other populations showed similar values ($h = 0.2280 – 0.2405$, $I = 0.3461 – 0.3640$).

Besides, five individuals of the subpopulation ESC172vb presented the same pattern of band presence/absence.

The NJ tree (Fig. 2) and the UPGMA dendrogram (not shown) generated by MEGA based on the Φ_{ST} pairwise difference matrix, as well as the UPGMA created by TFPGA (Fig. 3) based on the Nei's unbiased genetic distance matrix, show two main clusters. One is composed only by the ESC172 population, with two subclusters represented by the two flowers colors (light violet – ESC172vb and light yellow – ESC172a) and the second consists of two subclusters, with the first subcluster consisting of ESC173 and ESC195 populations, and the second subcluster consisting of ESC174 and ESC208 populations. This corresponds to the results of the Nei diversity (*h*) and the Shannon diversity (*I*), indicating that ESC173, ESC174, ESC195 and ESC208 are more similar among them than ESC172 when compared to each other. Besides, there was a high correlation between both matrices ($r = 0.798$, $P < 0.01$).

AMOVA generated Φ statistics, one analogous to Wright's F statistic. These analyzes reveals that 22.97% ($P < 0.001$) of the variation resided among populations within groups, a high proportion of the variance (51.72%, $P < 0.001$) resided within populations and that the differentiation between groups was 25.31% but not significant ($P = 0.0772$). Considering there was no significant difference between groups, an additional AMOVA analysis was performed to evaluate the variance components among and within populations. According to this partitioning 35.28% ($\Phi_{ST} = 0.3528$, $P < 0.001$) of the total variability was attributable to differences among population and 64.72% resided within ($\Phi_{IS} = 0.6472$, $P < 0.001$) (Table 4). The Mantel test using geographic distances and

Nei's (1978) unbiased genetic distance matrix found that they are not significantly correlated (r = 0.2663, P = 0.2204).

The data resulting from Bayesian genetic structure analysis of *Sisyrinchium micranthum* in the PEI enabled some insights about the gene flow and population subdivision. The model of k = 5 was the best to explain the clustering. The decision was made based on the probability of the data (ln prob) for each tested k value and its variance after ten iterations for each k. The distribution of ancestry coefficients Q of individual plants in each cluster is given in Fig. 4 and the average admixture proportion for each population among the clusters is shown in Table 5. Population ESC172 appears divided into two clusters and populations ESC173 and ESC195 are clustered together. The graph reveals that just a few individuals from populations ESC172a, ESC173, ESC174 and ESC195 are admixed with alleles from subpopulation ESC172vb and ESC208.

In relation to the chromosome number analysis, not all the collected samples presented flower buds at adequate stages for cytological studies. Diakinesis was the best phase for counting since the chromosomes are very small. Thus, it was possible to analyze the chromosome numbers for populations ESC172, ESC173 and ESC208. Table 6 presents the populations studied and their respective chromosome numbers. Subpopulation ESC172a presented 24 bivalents in microspore mother cells (MMC`s) (in diakinesis), so the somatic number is 2n = 48 (Fig. 5A). The other two populations analyzed exhibited an unexpected number, 8 bivalents, what corresponds to 2n = 16 (Fig. 5B). Diploid plants of *Sisyrinchium micranthum* were not mentioned in the literature this

being the first report. The numbers found might justify the differences among these populations and ESC172.

DISCUSSION

To study the distribution of the genetic variation within the populations of *Sisyrinchium micranthum* in the PEI, Nei's (1973) gene diversity (*h*), Shannon diversity (*I*), and the clustering methods based on Nei's (1978) genetic and pairwise genetic distances, as well as the $\Phi_{ST,}$ were calculated based on ISSR primers. The data shows that the populations are highly structured (Φ_{ST} = 0.3076). The Bayesian clustering result in addition to the high Φ_{ST} value, indicate a very low gene flow among populations.

The results suggest that in all analyses population ESC172 showed a different pattern. It was genetically less variable than the other populations and although the chromosome counts were performed in a single plant, possibly this population is composed by hexaploid individuals what might justify its isolated clustering. It should be mentioned that the hexaploid plants seemed to be morphologically smaller than those of ESC173, ESC174, ESC195 and ESC208 populations. However, exsiccates did not permit taking the morphology into account because only one individual was collected per site.

The geographic distances were not correlated with the genetic distances and could not explain the differentiation among collected sites. The variance within the populations explains 64.72% of the variation that corresponds well to the genetic structure of outcrossing plants. Usually, outcrossing populations have

more total genetic variation and less differentiation among populations than do the selfing plants.

High levels of intrapopulational variability have been correlated with the breeding system in other species. Holtsford and Ellstrand (1989), using isozymes, found that in the annual herb *Clarkia tembloriensis* Vasek (Onagraceae) there is a strong influence of the breeding system upon the distribution of the genetic variation within and among its populations. In this case, outcrossing populations had more total genetic variation and a lower differentiation among populations than the group of selfing plants. *Erodium paularense* Fern.Gonz. & Izco (Geraniaceae) is an outbreeding species and is endemic in Spain. Population genetics about this species showed that about 80% of the total genetic diversity was attributed to intrapopulational variation which agrees with the population structure of allogamous plants (Martín et al., 1997).

Truylio et al. (2002) studied the flower biology of *Sisyrinchium micranthum* in São Francisco de Paula, RS, located in southern Brazil, and found that this species is protogynous since female flower receptivity begins six to seven hours before male anthesis. Furthermore, controlled pollination experiments indicated that *S. micranthum* is self-incompatible. The pollinators are oil-bees from the bee family Apidae, tribe Tapinotaspidini (Cocucci and Vogel, 2001; Truylio et al., 2002), that are used to nesting close to the foraging area. However syrphids and small pollen-collecting bees were already seen visiting the flowers of *S. micranthum* and also of *S. vaginatum* Spreng. (Truylio et al., 2002; Freitas and Sazima, 2006). Thus, there is evidence suggesting that the low gene flow

among the populations may be due to the behavior of the probable pollinators. They are small insects that do not have a great ability to fly long distances, nor reasons to spend energy looking for more resources if there is enough in one foraging area.

The dendrograms obtained (Fig. 3) using the distance methods put together populations ESC173, ESC174, ESC195 and ESC208 in one of the two main clusters. The chromosome counts revealed that populations ESC173 and ESC208 have n = 8, which corresponds to a diploid chromosome number ($2n$ = 16). Since these populations clustered with the ESC195 and ESC208 it is possible that both are diploids. The chromosome numbers previously described for this species are $2n$ = 32 and 48 for individuals collected in North America (Goldblatt, 1982; Kenton and Heywood, 1984; Goldblatt and Takei, 1997). Kenton et al. (1986) studying *Sisyrinchium micranthum* mention a probable basic chromosome number of x = 8. Therefore, these individuals with $2n$ = 16 are diploids and ESC172 is hexaploid (n = 24).

Some aspects need to be highlighted. Firstly, why was population ESC172 subdivided into two (subclusters) even in the Bayesian analysis? It was not possible to know the chromosome number of subpopulation ESC172vb (light violet), since the buds were not in meiotic phases that allow chromosome counts (diakinesis). Nonetheless, the clustering methods of NJ and UPGMA suggest that both subpopulations are hexaploid since they remained in the same main cluster. Seven of 80 loci (about 9%) presented a different pattern of band presence/absence only in ESC172. While 13 (light violet flowers) of the 25 individuals presented one of these loci, the other 12 (light yellow flowers) did not

have the amplification mark and the opposite did happen as well. The other four accession areas did not have yellow flowers. Usually, fields with large populations of *Sisyrinchium micranthum* can be seen as a big light violet “rug” and the other colors can only be seen by walking in the field. So, the yellow flowers are not as common as the violet flowers, but in this population the proportion was about 1:1 of light yellow flowers and light violet flowers. Perhaps these loci are somehow related to the inheritance of flower color. The only doubt is: why did not the other populations (with light violet flowers) present the same band pattern as subpopulation ESC172vb had? One hypothesis is that polyploidy may have changed the inheritance of this trait, whatever it is, enabling an increased frequency of the yellow flowers and a consequent subclustering in the dendrograms according to the color of the subpopulations. The inheritance of flower color has been investigated in some species and until now it is not possible to generalize the results found in one species to other plants. Some species present a simple Mendelian inheritance and the flower color seems to be controlled by a single diallelic locus, like in the herbaceous aquatic perennial pickerelweed (*Pontederia cordata* L., Pontederiaceae) (Gettys and Wofford, 2007) and in morning glory [*Ipomoea purpurea* (L.) Roth, Convolvulaceae] (Zufall and Rausher, 2003). Nevertheless, other species have a more complex inheritance as in the case of *Petunia hybrida* Vilm. (Solanaceae). This species has a combined inheritance of anthocyanin pigmentation and vacuolar pH (Griesbach, 1996). In relation to the genus *Sisyrinchium*, in Japan the introduced *S. rosulatum* E.P. Bicknell shows two flower colors, white and purple. Investigations about flower color variation

indicate that white is dominant over the purple by a single Mendelian factor. According to the chromosome basic number of the genus, $x = 8$, this is a tetraploid species ($2n = 32$) (Yamaguchi and Hirai, 1987). It is not known whether the flower color inheritance of the other species of *Sisyrinchium* follows a Mendelian heritage, but Ramsey and Schemske (2002) in a review about neopolyploidy in flowering plants considering agricultural or horticultural cultivars and classic genetic systems, as *Datura* L. and *Nicotiana* L., cited that segregation ratios in autopolyploids and allopolyploids usually correspond to the multisomic inheritance model. The observations about the hexaploid population investigated here do not agree with this idea. But this question could be better exploited through crossing experiments.

The diversity indices of ESC172 were the lowest compared to the other populations ($h = 0.1496$, $I = 0.2237$). What is the relation among polyploidy and these estimates, and with the color pattern of the flowers? Is the breeding system somehow related to these aspects? Henderson (1976) studied *Sisyrinchium* species from North Hemisphere and reported a correlation between breeding system and ploidy level. Hand-selfing procedures showed that tetraploids are self-incompatible, while most higher polyploids are self-fertile. Besides, anthesis observations detected that protandry (maturation of the anthers before the stigma) is often present in blue-eyed grasses promoting outcrossing even in self-compatible plants. But, he observed that the higher the ploidy level the smaller the protandrous condition due to a decrease of the time interval between anthers and stigma maturation according to an increase in the ploidy level. Thus, while tetraploids were protandrous, most octoploids and

dodecaploids presented a short or absent maturation time interval promoting higher levels of self-compatibility and self-pollination as well, in the high polyploids plants. This might be the case of *S. bermudiana*. This species occurs over a wide area of North America, it is self-fertile and is divided into a large range of chromosome numbers (2*n* = 32, 64, 96) (Kenton et al., 1986; Ingram, 1968). Its flowers are protandrous, because anthers dehisce before the flower opens and after opening the stigma matures. Besides, the anther filament tube presents variation in its length. Hence, when the anther is at the same level of the style, there is a high probability of occurrence of self-pollination. Notwithstanding, outcrossing may occur when the filament tube is shorter than the style (Ingram, 1968). Nevertheless, the author did not investigate the relation between ploidy level and self-pollination.

The hexaploid acession in the PEI presented the lowest values of genetic diversity. Considering that polyploidy can cause changes in the breeding system, as mentioned above, this low genetic variation could be a consequence of selfing. Thus, this population would have a different breeding system compared to the other five sampled areas that are expected to be diploid and, mainly, self-incompatible according to the genetic diversity estimations, the variance component analyses and the related literature. But one question remains, the protogynous condition. As in the other species this characteristic could have been broken down as a consequence of the polyploidy process. This way the outcrossing would no longer be favored and self-pollination could occur. Hence the proportion of the flower colors may be related to the origin of this population and to the unknown self-fertilization index.

Besides, five specimens of the subpopulation ESC172vb presented the same band profile suggesting they are the result of self-fertilization or even clones, thus, the asexual reproduction may be contributing to the maintenance of this population. This is the first study based on genetic variability that indicates this species could not be based strictly on sexual reproduction. If this population reproduces vegetatively, how would it be associated with the mating system? Vallejo-Marín and O'Brien (2007) studied the relation between mating system and life history (annual/perennial) in *Solanum* L. (Solanaceae) and found a high correlation between self-incompatibility and clonality. The result of their work showed that all the self-incompatible plants were clonal and all strict annuals were self-compatible. However, clonality can increase the selfed offspring through geitonogamy (selfing through pollen transfer between flowers on the same plant) in *Decodon verticillatus* (L.) Elliott (Lythraceae) (Eckert, 2000). Thus, the low diversity indexes of ESC172 individuals can be a result of self-fertilization alone, or indeed, the consequence of clonality and selfing. How life history is connected to these aspects is a major doubt because *Sisyrinchium micranthum* is usually cited as annual (Johnston, 1938; Innes, 1985; Goldblatt, 2003), but according to Parent (1987) there were specimens in the Northwest of Spain that lived more than one year or, at least survived the winter.

Although it is not known undoubtedly whether something similar has been occurring in *Sisyrinchium micranthum* in the PEI (and how long), this could be an interesting mechanism to maintain the population in case of a pre or post-zygotic barrier between the hexaploids and the diploids in the beginning of a colonization. Thus, the decreased level of differentiation among individuals of

the ESC172 population possibly is a consequence of an initial population formation by a few polyploid specimens and the mating system. This founder effect and the reduced genetic variability might be related to the proportion of the flower colors in this population as well.

Considering that only one of the five accessions areas in the PEI presented *n* = 24, it could be suggested that the diploid individuals are more common than the hexaploids. The chromosome numbers described for this species are 2*n* = 32 and 48 for individuals collected in North America (Goldblatt, 1982; Kenton and Heywood 1984; Goldblatt and Takei, 1997). There are no studies in South America about this plant in spite of its wide distribution. It is not known whether tetraploids also occur here and how the evolution of these different ploidy levels is connected. It is necessary to pay attention to these populations inside the conservation park and to investigate if the same genetic variation and chromosome numbers occur in other areas inside the PEI and if this diversification has been maintained over the years. The distribution, the cytogenetics and the genetic variation of this species in Rio Grande do Sul state must be studied and the differences among the populations inside and outside the conservation park must be evaluated. This work has provided new insights about *Sisyrinchium micranthum.* Investigations of population genetics, besides describing the existence of diploid individuals for the first time were very important to raise more issues on the diversification of this plant. To carry out further research it is important to link both approaches, mainly because studies on *S. micranthum* are considerably scarce.

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Table 1. Data about collected populations of *Sisyrinchium micranthum* in Itapua State Park (Parque Estadual de Itapuã - PEI) in Viamão, Rio Grande do Sul, Brazil.

Population (Voucher)	Sub-population	Sampling site	Latitude (°S)	Longitude (°W)	N° individuals	Flower color
ESC172	ESC172vb	Praia de Fora	30°23'09.4''	51°01'07.2''	13	light violet
	ESC172a				12	light yellow
ESC173		Praia de Fora	30°22'58.5''	51°01'02.3''	17	light violet
ESC174		Pedra da Visão	30°21'57.2''	51°01'45.9''	27	light violet
ESC195		Morro da Grota	30°21'44.0''	51°01'13.0''	23	light violet
ESC208		Praia da Pedreira	30°21'30.4''	51°02'48.5''	26	light violet
Total					118	

Table 2. ISSR primers used, total number of fragments scored for each primer and the size of the amplified fragments.

Primer	Sequence (5 '- 3')	N° of recorded markers	Size range (bp)
P_2	$(GA)_8T$	13	400 - 1800
P_3	$(CTC)_4RC$	16	450 - 1634
P_4	$(CT)_8G$	9	480 - 1534
F_3	$(AG)_8C$	14	350 - 1434
F_4	$(GA)_8C$	14	325 - 1684
F_{11}	$(GACA)_4$	13	550 - 1634
Total		80	

Table 3. Results of single population genetic analyses of loci from five sampled populations of *Sisyrinchium micranthum* with six ISSR primers.

Population	N° polymorphic loci	Percentage of polymorphic loci	Nei diversity (*h*)	Shannon index (*I*)
ESC172	35	43.75	0.1496	0.2237
ESC173	58	72.50	0.2280	0.3461
ESC174	60	75.00	0.2405	0.3640
ESC195	63	78.75	0.2313	0.3532
ESC208	60	75.00	0.2339	0.3535

Table 4. Analyses of molecular variance (AMOVA) for *Sisyrinchium micranthum* populations using ISSR primers, partitioning the genetic variation into within and among populations.

Source of variation	df	Sum of squares	Variance	Variation (%)	Φ statistics	*P*
Among populations	4	441.280	4.360	35.28	$\Phi_{ST} = 0.3528$	< 0.001
Within populations	113	903.746	7.998	64.72	$\Phi_{IS} = 0.6472$	
Total	117	1345.026	12.358			

Table 5. Average admixture proportion for the *Sisyrinchium micranthum* populations (rows) in the Itapua State Park (Parque Estadual de Itapuã – PEI) among each of five genetic clusters (columns).

Population	Cluster				
	I	II	III	IV	V
ESC172	0.519	0.003	0.006	0.461	0.012
ESC173	0.044	0.011	0.873	0.028	0.044
ESC174	0.015	0.932	0.016	0.009	0.028
ESC195	0.010	0.053	0.887	0.011	0.040
ESC208	0.007	0.024	0.029	0.007	0.933

Table 6. Chromosome numbers observed in Microspore Mother Cells (MMC`s) of three *Sisyrinchium micranthum* populations of Itapua State Park (Parque Estadual de Itapuã – PEI).

Population/Sub-population	MMCs in diakinesis[1]	Chromosome number (*n*)
ESC172a	30 (1)	24
ESC173	40 (1)	8
ESC208	45 (2)	8

[1] Number of analyzed individuals is in parenthesis.

Fig. 1. (**A**) Map of South America; (**B**) Itapua State Park (Parque Estadual de Itapuã - PEI) and the five study populations of *Sisyrinchium micranthum* – ESC172, ESC173, ESC174, ESC195 and ESC208. Bar = 1 km.

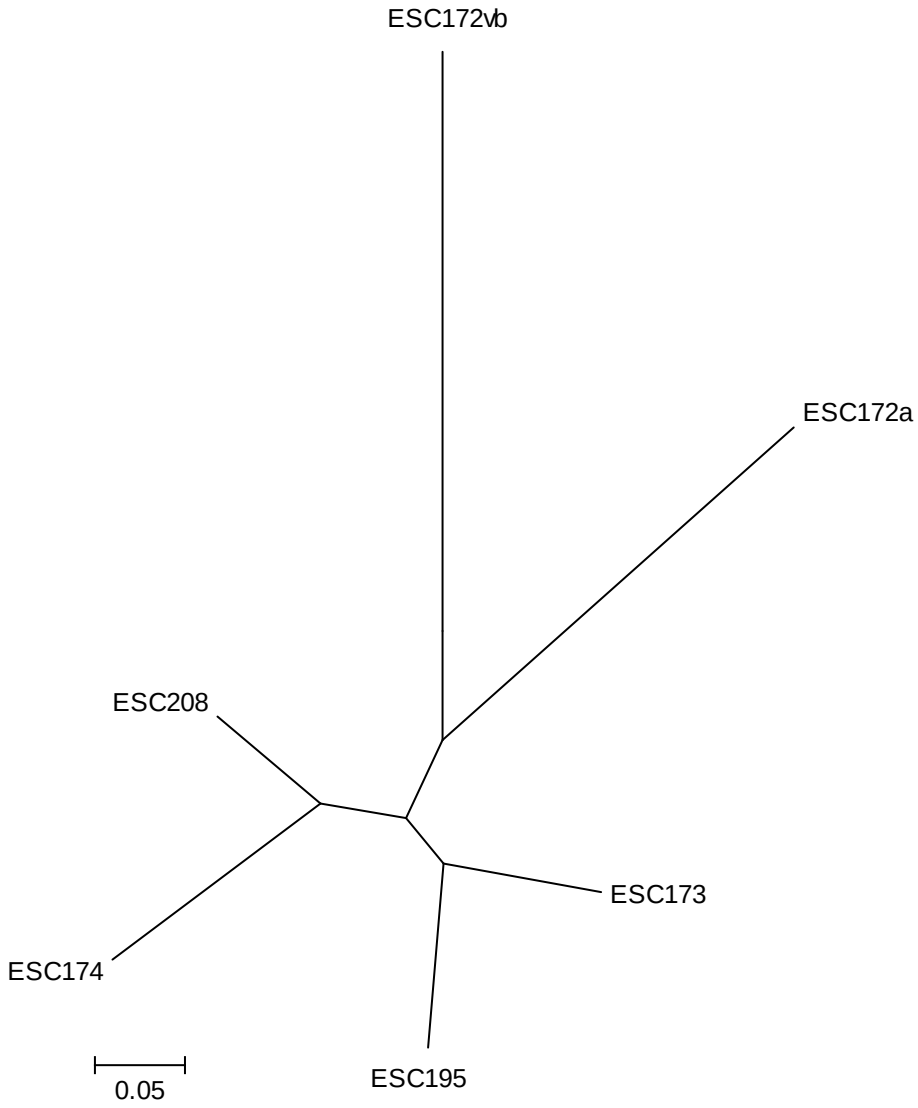

Fig. 2. Unrooted neighbor-joining (NJ) tree based on the Φ_{ST} pairwise difference matrix of five studied populations of *Sisyrinchium micranthum* from Itapua State Park (Parque Estadual de Itapuã – PEI): ESC174, ESC208, ESC173, ESC195 and population ESC172 and its subpopulations ESC172vb and ESC172a. A scale for genetic distance is provided at the bottom of the graph.

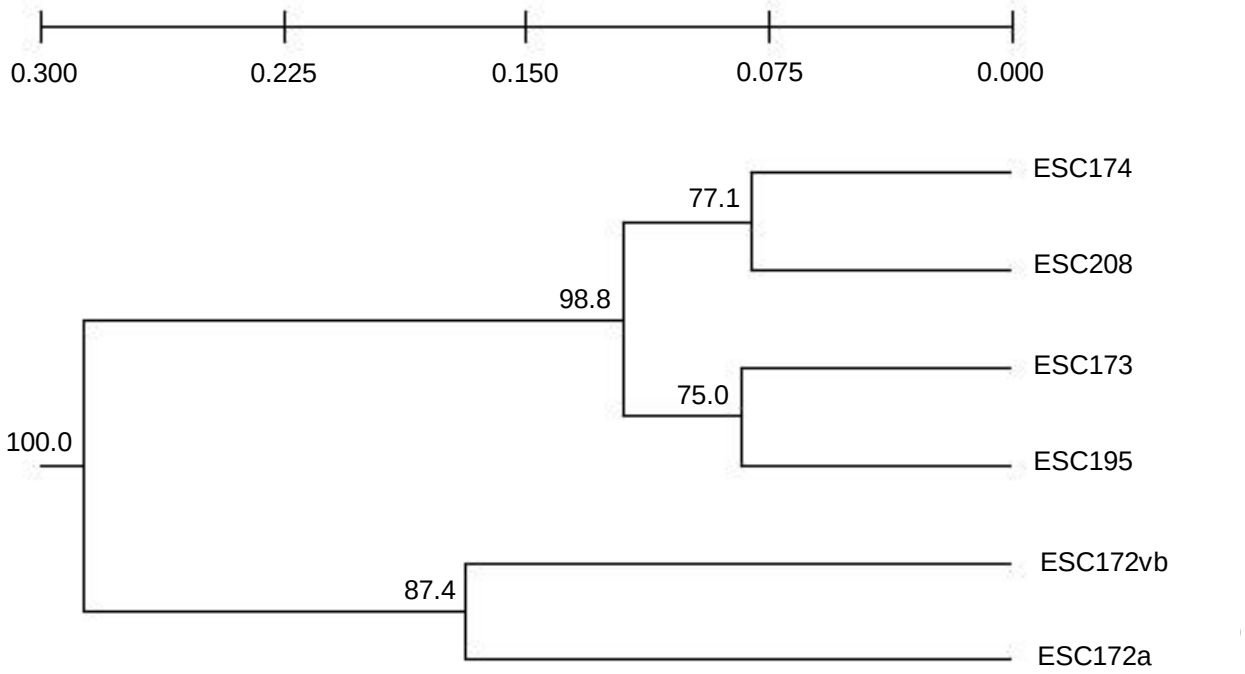

Fig. 3. Unweighted pair-group method arithmetic average (UPGMA) based on Nei (1978) genetic distance, including bootstrap support values in percent, of populations of *Sisyrinchium micranthum* present in the Itapua State Park (Parque Estadual de Itapuã – PEI): ESC174, ESC208, ESC173, ESC195 and population ESC172 and its subpopulations ESC172vb and ESC172a. A scale for genetic distance is provided at the top of the graph.

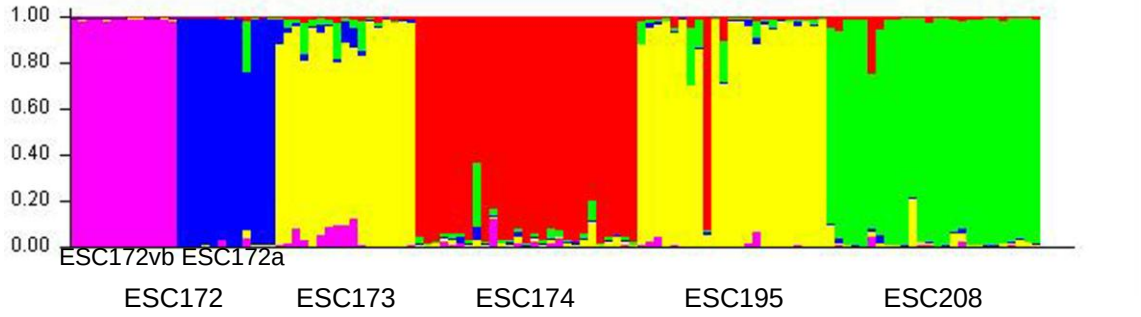

Fig. 4. Bayesian admixture proportions (*Q*) of individual plants of *Sisyrinchium micranthum* for *k* = 5. Each individual is represented by a single vertical line broken into *k* colored segments, with lengths proportional to each of the *k* inferred clusters. Accessions from Itapua State Park (Parque Estadual de Itapuã – PEI): ESC172, ESC173, ESC174, ESC195 and ESC208.

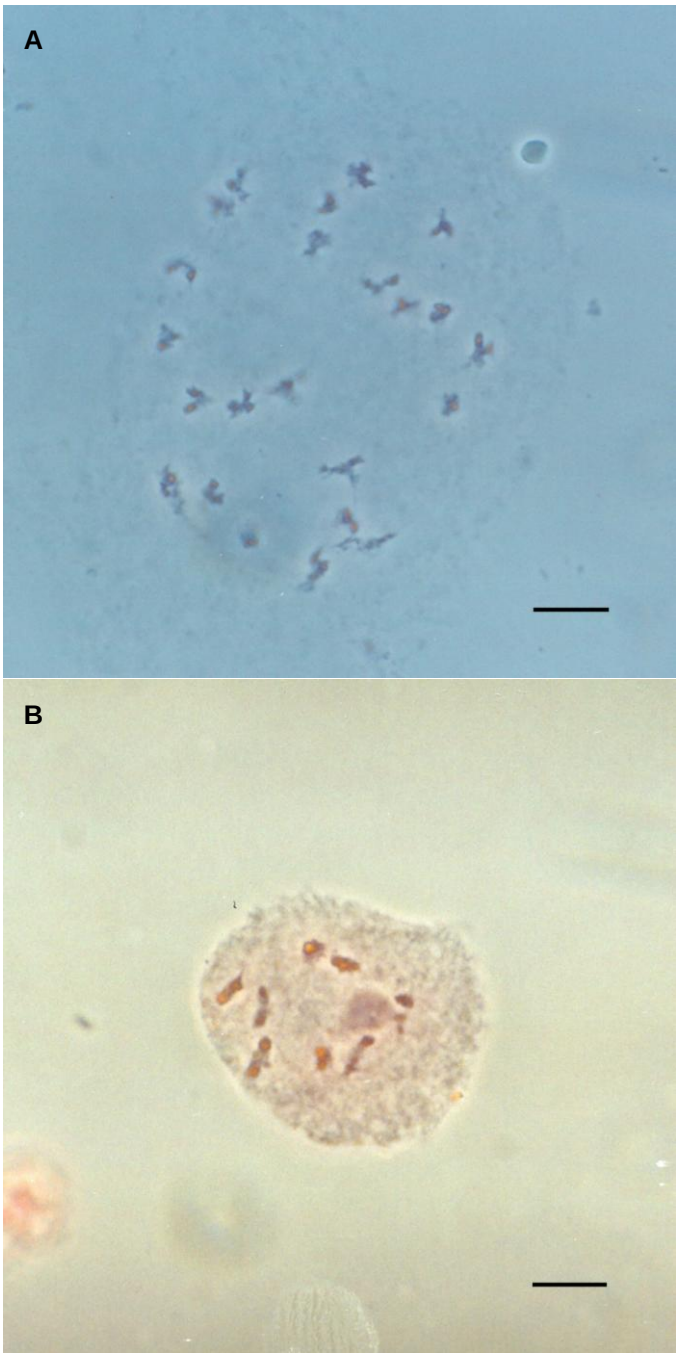

Fig. 5. Chromosome numbers of *S. micranthum* in the Itapua State Park (Parque Estadual de Itapuã – PEI). (**A**) Diakinesis with 24 bivalents (*n* = 24); (**B**) Diakinesis with 8 bivalents (*n* = 8). Bar = 10 µm.

MORPHOLOGICAL VARIATION IN *SISYRINCHIUM MICRANTHUM* CAV. (IRIDACEAE) OF SOUTHERN BRAZIL: CYTOGENETICAL AND MOLECULAR APPROACHES

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MORPHOLOGICAL VARIATION IN *SISYRINCHIUM MICRANTHUM* CAV. (IRIDACEAE) OF SOUTHERN BRAZIL: CYTOGENETICAL AND MOLECULAR APPROACHES[1]

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Sisyrinchium L. is recognized as a highly variable genus of Iridaceae and its taxonomy is notoriously confused. *Sisyrinchium micranthum* Cav. presents a wide distribution on the American continent, and in the South of Brazil field observations have allowed to verify the existence of a wide variation in plant size and flower color. It is possible to find *short*, *medium* and *tall* plants, and violet, pink and yellow flower colors. The chromosome numbers described in the literature for *Sisyrinchium micranthum* are 4*x* = 32 and 6*x* = 48, in relation to the specimens collected in the Northern Hemisphere, and diploid (2*x* = 16) and hexaploid (6*x* = 48) individuals has been identified in the South of Brazil. Investigations on meiotic behavior, analysis of pollen fertility and genetic variability, together with the characterization of morphological types can help explain the taxonomic and evolutionary relations of the species. In this study the different morphological types mentioned were evaluated as to cytogenetic and molecular aspects. The cytogenetic and molecular data indicate that *S. micranthum* presents populations with more than one level of ploidy, showing the wide variation of the morphological characters which makes it difficult to identify and classify the plants. The existence of 2*x*, 4*x* and 6*x* individuals was verified, but no direct relationship was detected between level of ploidy and morphological traits of the plants. The *medium* type is most frequent in southern Brazil and its chromosome number is generally 2*x* = 16. Most plants examined had regular meiotic behavior and all morphological types presented high estimations of meiotic indexes and pollen viability. These cytogenetic, molecular and morphological data are new and show the importance of maintaining these

populations in the South of Brazil, in order to preserve this variability and continuity in the evolutionary process of the species.

Key words: genetic variability; Iridaceae; ISSR-PCR; meiotic behavior; pollen viability; reproductive biology; *Sisyrinchium micranthum*

The family Iridaceae is a relatively large one among the monocotyledon plants, with about 1900 species and 65 genera (Goldblatt and Manning, 2006). It is widely distributed throughout the world, and is concentrated mainly in the Southern Hemisphere, Africa being the center with the greatest diversity (Goldblatt, 1990).

Sisyrinchium micranthum Cav. belongs to the family Iridaceae, subfamily Iridoideae, tribe Sisyrinchieae and is characterized by linear leaves, flattened floral stems and terminal inflorescences with cupped flowers that might show a great variety of colors, such as white, yellow, lilac and others (Chukr and Capellari Jr., 2003). In these plants, filaments are united over about 1/2 to 2/3 of their length, with great density of elaiophores at the base. The species occurs between Mexico and Argentina, besides occurring in a naturalized form in Australia, Malasia and the Fiji Islands (Innes, 1985). In Brazil it is distributed over Rio Grande do Sul, Santa Catarina, Paraná, São Paulo and Rio de Janeiro, inhabiting different environments such as grasslands, open woods or anthropogenic sites (Chukr and Capellari Jr., 2003). Normally it is referred to be annual (Johnston, 1938; Innes, 1985; Goldblatt, 2003).

The species presents a great variation in size of the individuals (Chukr and Capellari Jr., 2003). This variation in size was observed in Rio Grande do Sul (RS), Brazil, and in the field expeditions *short*, *medium* and *tall* plants were recognized. There is also a notable variation in color of flower, with predominance of violet, pink and yellow flowers. Studies have been performed to establish morphological categories of *S. micranthum* plants present in the South of Brazil.

Sisyrinchium L. is recognized as a highly variable genus (Henderson, 1976) and its taxonomy is notoriously confused (Cholewa and Henderson, 1984; Kenton et al., 1986; Rudall et al., 1986) due to the difficulty of finding safe taxonomic characters since usually the characters taken into account are those that present a wide grading of expression in some populations (Henderson, 1976).

Of the 1900 species that belong to the family Iridaceae, chromosome number of slightly over 1000 has already been determined and these are mostly species from the Northern Hemisphere and Africa (Goldblatt and Takei, 1997). Cytological information regarding the Iridaceae species that occur in South America is really scarce, above all for the Sisyrinchieae tribe (subfamily Iridoideae) (Goldblatt, 1982; Kenton et al., 1986).

Although *Sisyrinchium* is the largest genus in this tribe, the cytogenetic studies are restricted for species from North America (Ingram, 1968; Henderson, 1976; Cholewa and Henderson, 1984; Kenton et al., 1986; Goldblatt and Takei, 1997). The genus is described as having small chromosomes and high chromosome numbers which are quite variable due to polyploidy and hybridization (Kenton et al., 1986).

The chromosome numbers described in the literature for *Sisyrinchium micranthum* are $2n = 32$ and $2n = 48$, from specimens collected in the Northern Hemisphere, and the probable basic number is $x = 8$ (Goldblatt, 1982; Kenton and Heywood, 1984; Goldblatt and Takei, 1997). Diploid individuals, $2n = 2x = 16$, were described for the first time in a study on the genetic variability of *S. micranthum* in a conservation unit in the south of Brazil (in prep.). In the

aforementioned study, five populations of this species were studied, in which one presented 2*n* = 6*x* = 48 and two other populations presented 2*n* = 2*x* = 16.

Basic studies such as on pollen viability are also rare, even for the genus. A study on the reproductive biology of *Sisyrinchium vaginatum* Spreng. in southeastern Brazil, showed high pollen fertility, varying from 88% to 96%. Despite the importance of analysis of meiotic behavior and pollen fertility in studies on taxonomy, genetic variability, biodiversity and evolutionary biology, so far there has been no research using these approaches in *S. micranthum*. Published cytogenetic studies are limited to chromosome counts.

Studies using nuclear molecular markers to investigate the genetic variability of species of the family Iridaceae are also scarce (Wróblevska et al., 2003; Caiola et al., 2004; Meerow et al., 2005; Meerow et al., 2007). Additional studies analyzing the population variability of *Sisyrinchium micranthum*, and chromosome counts have already been accomplished (in prep.). The results showed that the use of ISSR primers may be an auxiliary method to relate ploidy level and genetic diversity, emphasizing the importance of using molecular and cytogenetic tools to characterize a species and to ask questions about their evolutionary history. To analyze the genetic diversify ISSR (Inter Simple Sequence Repeats), is a PCR-based (Polymerase Chain Reaction) technique widely used and it involves the amplification of a DNA segment present at an amplifiable distance between two regions with identical microsatellites oriented in opposite directions. Primers of 16 to 25 pb are used, constituted by di, tri, tetra or pentanucleotidic sequences.

Due to the scarcity of studies on *Sisyrinchium micranthum* and the wide morphological variability presented by the plants in the South of Brazil, the purpose of this paper is to characterize different morphological types as to cytogenetic and molecular aspects, which allow inferences about their taxonomic and evolutionary relations, as well as the breeding system of the species.

MATERIAL AND METHODS

Sisyrinchium micranthum plants were collected from a total of twenty-five sampling sites in Rio Grande do Sul, southern Brazil (Fig. 1). The individuals were collected and classified in the field according to the morphological types cited in Introduction. Table 1 shows the names, localities and coordinates of the sampling areas. Voucher specimens have been deposited in the herbarium of the Instituto de Biociências da Universidade Federal do Rio Grande do Sul (ICN).The individuals were characterized morphologically.

Cytological analyses

Inflorescences from at least ten individuals for each accession were collected and fixed in 3:1 ethanol:acetic acid for 12-24 hours at room temperature. After fixation, buds were transferred to 70% ethanol and stored in a freezer at -18°C.

Chromosome numbers, meiotic behavior and pollen fertility

Preliminary analyses demonstrated that pollen mother cell wall was very stiff making difficult to acquire a good chromosome spread. Thus, prior to analyses,

three anthers present in each flower were washed in distilled water and then hydrolyzed in pectinase-cellulase 2% at 37°C for 30 min. For slide preparation, the anthers were washed again in distilled water and squashed in 1% propionic carmine. Slides were examined and documented with a Zeiss Axioplan Universal photomicroscope.

All available phases of meiosis were analyzed. It was considered that there were abnormalities in meiosis when there was the presence of not aligned chromosomes on the metaphase I, and bridges and laggards at anaphase and telophase I and II. Meiotic indexes were calculated from 200 pollen tetrads per plant. The presence of microcytes and micronuclei, bridges and non equal-sized cells was considered abnormal.

Pollen viability was estimated for seven to 10 individuals per accession and 500 mature grains per plant. Squash preparations were stained following Alexander's method (1980).

Pollen and anther measurements

Nineteen pollen grains per individual, stained with Alexander's method (1980), were analyzed for the size of the largest (P) and smallest (E) axis. Based on these measures, area and P/E ratio of these grains were calculated. Since the grains of this species presented a shape similar to an ellipse, the area was calculated according to formula $A = \pi PE/4$. The P/E ratio was used to classify the morphological types as to pollen grain shape, according to Erdtman (1971). In order to characterize the morphological types, each anther used to prepare the slides, both for meiosis and pollen analysis, was measured for length and

related to the cellular division phase of the reproductive cells (meiosis I and II, tetrad and pollen). The measurements were performed using a stereomicroscope.

Statistical analysis

To compare the morphological types in relation to the pollen measurements (longer axis, shorter axis and area) ANCOVA (Analysis of Covariance) test was conducted using the length of anther as a covariate. The LSD (Least Significant Difference) test was used for multiple pairwise comparisons.

The anther measurements corresponding to the pollen cell division stage were statistically analyzed using the Kruskal-Wallis test. The anther measurements related to meiosis I and II, and tetrad stage were not statistically analyzed due to the small sample number of some morphological types.

Molecular analysis

Plant material

To analyze the molecular relationship among the morphological types in relation only to the size of *Sisyrinchium micranthum*, nine sampling areas were investigated using ISSR primers, three for each type. Thus the accessions corresponding to the *medium*, *short* and *tall* types are: ESC160, ESC175 and ESC176; ESC172, ESC177 and ESC197; and, ESC164, ESC170 and ESC198, respectively. Ten specimens with light violet flowers were collected from each sample. One individual of *S. palmifolium* L. was employed as outgroup (ESC132C).

DNA isolation and ISSR-PCR amplification

Total genomic DNA was extracted from 10-50 mg of silica gel-dried leaves according to the Doyle and Doyle method (1987) with modifications. Six primers that generated good patterns with a representative sample group were further used for amplification of plant DNA of all the sampling accessions. PCR reactions were carried out in 25 µl volume. The conditions had to be optimized for each primer and after the reactions were performed as follows (depending on the primer): 4% DMSO, 1x buffer, 4.6–5.0 mM $MgCl_2$, 0.48–0.8 mM of each dNTP (Invitrogen™ Brazil), 0.4–0.6 µM of each primer, 1 U *Taq* polymerase (CentBiot®/Brazil) and 10 ng of genomic DNA. Amplifications were executed using a Techne TC412 Termocycler with initial denaturation of 5 min at 92°C, followed by 35 cycles of 1 min at 94°C, 1 min at 42–45°C (depending on the primer Ta), 2 min and 30 seconds at 72°C and a 5-min final extension step at 72°C. PCR products were analyzed on 1.5% agarose gels and stained with GelRed™.

Cluster analyses

Bands were scored for presence (1) or absence (0), and a binary file was prepared. Similarity matrices based on Jaccard and Dice indexes were generated to create a dendrogram using an unweighted pair-group method arithmetic average (UPGMA). Cophenetic correlations were calculated to measure goodness of fit for both matrices. A Mantel test was performed to calculate the degree of positive correlation between Jaccard and Dice matrices. All analyses were carried out with Ntsys 2.1 (Rohlf, 2000).

Results

Morphological characterization

The *short* and *medium* plants are very similar in habit, stems and leaves, but different in size, with *short* plants presenting a markedly smaller vegetative aspect and flowers that are about half the diameter of *medium* type flowers. Besides this aspect, they often present broader flower cup than plants of *medium* type. The *tall* morphological type is characterized by presenting the most impressive differences. The habit of the plants is obviously more erect than the previous types and spathes are longer, narrower and closer to each other. The *tall* type flowers also present the flower cup with a very characteristic yellow color bordering it. Another aspect that should be emphasized is that this morphological type occurs preferentially in the Campos de Cima da Serra region, where the largest populations were observed. This region is characterized by presenting the greatest altitudes and rainfall levels in the State.

The *medium* morphological type is most widely distributed in the State. It occurs in dense populations in several regions of Rio Grande do Sul.

Cytological analyses

Table 2 shows the chromosome numbers, meiotic behavior and pollen viability for each morphological type in different accessions. Some accessions did not present flower buds in all the developmental stages desired (in meiosis I and II, tetrads and mature pollen). The small size of the chromosomes in *Sisyrinchium micranthum* and the difficulty of spreading them allowed the counts only in the

diakinesis stage. Furthermore, when a slide obtained from squashing and staining a floral bud with meiotic cells presented cells in the prophase stage, cells in metaphase I or other later division stages were not found in it. Thus, in the accessions with more than one level of ploidy, it was not possible to relate the analyses of irregularities in meiosis I and II, tetrad and pollen viability to their respective chromosome numbers. Therefore, in these cases the analyses refer strictly to the accessions as a whole.

All morphological types of *Sisyrinchium micranthum* had the chromosome numbers determined resulting in 12 accessions analyzed. In spite of the chromosome counts were realized in meiotic cells, the discussion will be made concerning the diploid number to get a clear view of the ploidy levels. Three different cytotypes were found: $2n = 2x = 16$, $2n = 4x = 32$ and $2n = 6x = 48$ (Fig. 2). *Medium* light violet plants presented $2n = 2x = 16$ (diploids) or $2n = 4x = 32$ (tetraploids). Plants with the phenotype *medium* pink, *medium* yellow and *tall* light violet were $2n = 2x = 16$, while the *short* light violet was $2n = 4x = 32$. Only the *short* light yellow type presented $2n = 6x = 48$ (hexaploids). Therefore, all the populations studied have the basic chromosome number $x = 8$. The most common chromosome number among the sampled accessions was $2n = 2x = 16$. Tetraploids were described for the first time in Brazilian populations. Tetraploid individuals were found for *medium* or *short* phenotype. The difficulty of separating these morphological types is outstanding, especially because they possibly occur mixed in the same field.

The results presented in this work are the first record on meiosis for *Sisyrinchium micranthum*. Meiotic behavior was examined for 13 accessions

covering all the chromosome numbers and all the morphological types (Table 2). Most plants examined had regular meiotic behavior with less than 20.0% abnormalities in meiosis I and II. No presence of multivalents was found in any of the accessions analyzed. All morphological types presented a total of meiotic indexes and pollen viability higher than 86% and 87%, respectively. Nevertheless, some accessions presented higher levels of abnormalities in meiosis I and/or II, or showed lower frequencies of meiotic indexes or pollen viability compared to the majority, regardless of their chromosome numbers. But it should be mentioned that all the sampled polyploids showed higher levels of irregularities in some of the analyses, with the exception of ESC197, *short* light violet, 2*n* = 32. Some abnormalities are shown in Fig. 3.

Due to the wide morphological variation in *Sisyrinchium micranthum* and the occurrence of polyploidy in this species, an association established between floral morphometric parameters and microsporogenesis stages make it easier to select adequate anthers to analyze meiosis, tetrads and pollen. This way, anthers were measured before the cytological analysis and meiosis phase was recorded. Pollen grains were also measured to investigate if there is any relation between pollen size and the chromosome number/ploidy level. Both analyses may help the characterization of the morphological types of *S. micranthum.*

The average anther length (Table 3) ranged from 1.90–3.53 mm for meiosis I and II stages, 2.20-4.10 mm for tetrad stage and 4.5 –7.71 mm for pollen analysis. Regarding the anther measurements for microspore stage a straight relationship was established between the anther height and the plant size. The

tall type presented a significant large anther size and the *short* type had the shorter size, while the *medium* type showed intermediate values. In relation to the other cell division stages a similar tendency was observed, but the samples sizes were small for some morphological types making statistical analysis unsuitable. Even so, the results suggest that the *short* type has the shorter means anthers, 1.90 and 2.33 mm.

The results of pollen measurements are shown in Table 4. Pollen grains of all the morphological types were classified as subprolate since then the P/E varied from 1.15 to 1.24 (according to Erdtmann, for subprolate pollen P/E varies from 1.14 to 1.33). The averages for pollen longer axis (P) varied from 30.73 to 36.35 µm and from 25.19 to 30.34 µm for the shorter axis (E).Considering the pollen area, the morphological types varied from 2 447.11 to 3 513.07 μm^2. The *short* light yellow presented higher measures of pollen longer axis (P), pollen shorter axis (E) and area. Two accessions were analyzed for this morphological type: ESC172 ($2n = 6x = 48$) and ESC161 (chromosome number undetermined). *Medium* light yellow (ESC160, ESC197 and ESC205), $2n = 2x = 16$, had the smallest pollen longer axis together with the *tall* light violet (ESC164, ESC170, ESC198 and ESC202), $2n = 2x = 16$. *Medium* light yellow also presented the lowest averages for the pollen shorter axis and area, followed by the *tall* light violet. The other morphological types had similar or overlapped results.

Molecular analysis

Amplifications of the six primers produced 93 scored loci with 98.92% polymorphism. The ISSR fragments generated an average of 15.5 bands per

primer. The size of amplified products ranged from 250 to 1 740 bp (Table 5). The percentage of polymorphic loci for each accession was: 56.99% for ESC160, 61.29% for ESC175, 54.84% for ESC176, 50.54% for ESC164, 48.39% for ESC170, 54.84% for ESC198, 45.16% for ESC177, 44.09% for ESC197 and 6.45% for ESC172.

In the UPGMA trees generated by NTSYS based on Jaccard (Fig. 4) and Dice (not shown) indexes resulted in good fit (r = 0.82 and r = 0.86, p = 0.001, for Dice and Jaccard, respectively) suggesting that the dendrograms are reasonable representations of the relationships among accessions. Moreover, the Mantel correlation between Dice and Jaccard matrices was high and significant (r = 0.99, p = 0.001). The ten accessions formed groups of individuals from each sampling area almost exclusively. Besides, it is possible to identify two main clusters. One is composed just by ESC175 (*medium* type, $2n = 2x = 16$) and ESC160 (*medium* type, $2n = 2x = 16$) in one subcluster and by ESC172 (*short* type) in another subcluster. The second main cluster is divided into two other subclusters. One of them is composed by the *tall* type with ESC164 (*tall* type, $2n = 2x = 16$), ESC170 ($2n = 2x = 16$) and ESC198 ($2n = 2x = 16$) and by ESC176 (*medium* type, $2n = 4x = 32$). But ESC164 and ESC176 were more closely related as well as ESC170 and ESC198. The other one is formed by ESC197 (*short* type, $2n = 2x = 32$) and ESC177 (*short* type). These data gathered with the cytological results suggest that a same accession can be composed by “mixed populations” in which different ploidy levels occur together. The accession ESC172 stands out because it had the lowest value for polymorphic loci with high indexes of similarity between the individuals (Fig. 4).

DISCUSSION

As mentioned previously, the populations of *Sisyrinchium micranthum* present a large morphological variability in Rio Grande do Sul (RS), Brazil, and the plants can be classified as different types: *short*, *medium* and *tall*, as to size, besides presenting variation in the flower color. Even within each morphological type there appears to be a variation in the vegetative and reproductive characters. Among the three morphological types, the most frequent as to size is the *medium* type. Considering the clear variation of plant size for this species, and the occurrence of polyploidy within the genus, it is asked whether this morphological variation could be related to the chromosome number, or whether it is a different species. Despite the clear morphological differentiation between types, it is not possible, initially, to consider them different species or subspecies.

The chromosome numbers already described in the literature for this species are $2n = 4x = 32$ and $2n = 6x = 48$, in relation to individuals collected in the Northern Hemisphere (Goldblatt, 1982; Kenton and Heywood, 1984; Goldblatt and Takei, 1997). More recently our group, studying populations in the Itapua State Park (Parque Estadual de Itapuã – PEI) (RS, Brazil) found the occurrence of hexaploid individuals ($6x = 48$) and, for the first time the existence of diploids ($2n = 2x = 16$) was recorded for the species (in prep.). However, no tetraploid individuals were found ($2n = 4x = 32$). In the present study, the cytological analysis proved the occurrence of tetraploid individuals in populations in the south of Brazil. Therefore, three levels of ploidy are found for this species in this region.

The data obtained from the 12 accessions analyzed suggest that the diploid individuals are more frequent in the State, since in 10 of the 12 areas accessed, this level of ploidy occurred. Furthermore, the results show that the tetraploid individuals were identified in the field, as well as *small* plants (ESC197), and *medium* (ESC166, ESC171 and ESC197), showing a grading in the variation of morphological characters.

The analyses of meiotic behavior and pollen fertility for this species constitute previously unknown data that is extremely relevant to understand the possible mechanisms involved in its evolutionary process. For most types and accessions, low frequencies of abnormalities were found, resulting in high meiotic and pollen viability rates, indicating that the plants are male-fertile. In general, independently of the level of ploidy, the plants analyzed presented meiotic stability. However, most of the accessions that presented the occurrence of polyploids resulted in a higher frequency of irregularities in at least one of the analyses performed.

These overall low frequencies of abnormalities in meiosis and high levels of pollen viability together with the non-occurrence of multivalents in none of the polyploid accessions analyzed may indicate a possible origin by allopolyploidy. However, it should be stressed that the chromosomes of this species are small in size and that the formation/maintenance of the multivalents may be rendered difficult due to the low frequency of chiasmata formed (Stebbins, 1971). If the region for pairing between the chromosomes is too short to allow the formation of more than one chiasma, it is unlikely that there will be multivalent formation. It is usually thought that autopolyploids have lower fertility than allopolyploids as a

consequence of an expected higher frequency of irregularities such as univalents and multivalents (Stebbins, 1950). Nevertheless, Ramsey and Schemske (2002) in an extensive revision on newly synthesized auto- and allopolyploids show the mean pollen viability of new polyploids is not significantly different, but it is significant reduced when compared to the parentals. Otherwise, extensive genome and chromosomal rearrangement may increase the fertility of newly formed polyploids leading to a process of diploidization (Soltis et al., 2003).

Henderson (1976) analyzed 75 Pacific Northwestern blue-eyed grasses populations and nearly all plants presented regular meiosis and high levels of pollen viability (95–100%). However, dodecaploids populations of *Sisyrinchium* from northwestern Canada showed some meiotic irregularities (four or five univalents at diakinesis) and lower pollen stainability (79–91%). Besides, these plants had morphological characteristics of *S. idahoense* E.P. Bicknell, *S. montanum* Greene and *S. septentrionale* E.P. Bicknell, thus the authors considered these could be interspecific hybrids. Cholewa and Henderson (1984) found dodecaploids specimens similar to *S. septentrionale* (often $n = 16$) in the same region that Henderson studied and cytogenetic analyses showed normal meiotic behavior, which, according to the authors, suggested the occurrence of stabilized hybrids. Considering the great morphological diversity in *S. micranthum*, allopolyploidy could be acting as the source of such variability and the actual meiotic behavior could not have a straight relation with the ploidy condition.

But, until now, morphological characteristics did not suggest any similarity among *Sisyrinchium micranthum* and other related species. Furthermore, observations in the field indicate that there are no populations of other species of *Sisyrinchium* occurring in sympatry with *S. micranthum*. And even when there are some exemplars of another species of *Sisyrinchium* occurring close, the floral anthesis period appears distinct. *Sisyrinchium palmifolium* and *S. vaginatum* are some of the species that have already been seen occurring in sympatry with *S. micranthum*. However, specimens of *S. palmifolium* from Argentina have already been classified as narrow afternoon blooming since their flowers start opening at 15:30 h (Cocucci and Vogel, 2001). Freitas and Sazima (2003) studied *S. vaginatum* in southeastern Brazil and verified that most flowers open between 13:00 and 15:00 h. But, *S. micranthum* is morning blooming according to a study performed in São Francisco de Paula, RS, Brazil (Truylio et al., 2002). Other reproductive aspects are also distinct, *S. micranthum* has elaiophores, special floral glands that produce lipidic oils, used by some pollinators, while *S. palmifolium*, *S. vaginatum* and most species of *Sisyrinchium* do not have these glands (Cocucci and Vogel, 2001). Beginning with this idea, an origin by interspecific hybridization does not appear very likely. However, this last aspect may even restrict the sharing of a few pollinators, but it may not constitute a barrier, since there are reports of generalist floral visitors such as pollen collecting bees or other insects such as syrphids for *S. micranthum* (Cocucci and Vogel, 2001; Truylio et al., 2002; Freitas and Sazima, 2006). Thompson et al. (2004), studying *Heuchera grossulariifolia* Rydb. (Saxifragaceae) and its relationship with herbivores and pollinators, obtained a

series of results that suggest that polyploidy may constitute an important factor in the structure and diversification of terrestrial communities. In this way the floral morphology and the plant-pollinator interactions are issues that need to be better investigated, both at a population level and in communities, for a better understanding of the evolution of *Sisyrinchium micranthum* and also of the other species that may be related to its reproductive processes.

Although this work is not for the purpose of elucidating the emergence and evolution of the different cytotypes of *Sisyrinchium micranthum*, a few speculations are possible. The tetraploid individuals could emerge from the union of non-reduced gametes of diploid individuals ($n = 16$ x $n = 16$). The emergence of hexaploids ($2n = 6x = 48$) may have several origins, whether it be from crossings between diploids with reduced and non-reduced gametes ($n = 8$ x $n = 16$), or from the crossing of diploids with tetraploids ($n = 8$ x $n = 16$), in both cases followed by chromosome duplication ($24 \rightarrow 48$). It could also have emerged from the union of reduced and non-reduced gametes of tetraploid individuals ($n = 16$ x $n = 32$). However, such scenarios for the origin of polyploids require the non-existence of barriers to crossing between cytotypes. It should be pointed out that individuals with 24 chromosomes or with 40 ($3x$ and $5x$, respectively) were not found, and this may indicate the current existence of barriers between cytotypes.

The fact that it was identified individuals with $2n = 2x = 16$ and $2n = 4x = 32$ in sympatry in more than one population could suggest that the emergence of the tetraploids has occurred recurrently, but at the same time in our samples there were no intermediate cytotypes, what does not support this idea.

Both the data on chromosome number, analysis of the microspore and anther size, and molecular analysis show that there are differences between the types, but their consistent separation was not possible, very likely due to the fact that the populations present diploid and tetraploid individuals in sympatry. Often the distinction in the field of the morphological types is quite complicated, because a few individuals present intermediate characters or share similarities with the *short* and *medium* types. Genome restructuring might represent a valuable source of genetic diversity in natural populations (Soltis and Soltis, 1999) and this might be the case of *Sisyrinchium micranthum.*

It was initially expected that the size of the plants, the size of the anthers and the pollen grains were directly related to the level of ploidy, since polyploids generally present *giga* characteristics, such as flowers, pollen grains and larger seeds (Ramsey and Schemske, 2002). However the population in which a hexaploid individual was found (ESC172, *short* yellow) was classified as *short* type, presented smaller anthers, but its pollen grains were significantly larger than those of the other accessions in all measurements performed. Meanwhile, the plants classified as *tall* type presented larger anthers, but its pollen grain measurements were one of the smallest and the chromosome counts resulted in $2n = 2x = 16$.

Heuchera grossulariifolia is an endemic plant from the North of Idaho in the United States, which presents populations of diploid and tetraploid individuals, occurring sympatrically. Throughout their occurrence in Salmon River, diploid and tetraploid plants differ morphologically, and it is possible to distinguish them easily with the naked eye. However, in other locations it is not possible to

identify consistent morphological differences between diploid and tetraploid plants in the field (Thompson et al., 2004).

These data indicate that the relationship between level of ploidy and vegetal morphology is not always direct and does not necessarily result in very expressive phenotypical traits. Performing an artificial crossing between individuals with different levels of ploidy in *Sisyrinchium micranthum* may help explain this issue. The crossing between the morphological types is also interesting, since the phenotypic variation may be due to different causes: pattern of inheritance, chromosome number, or else environmental influence (phenotypic plasticity). According to Goldblatt (1982) e Rudall et al. (1986), the level of ploidy increases with the latitude in species of *Sisyrinchium*: diploids occur in Mexico and the south of the USA, tetraploids in the north of the USA, and those with higher levels of ploidy (octa and dodecaploids) in Canada. The high levels of ploidy in the temperate zone of the Northern Hemisphere may indicate a need for an increased heterozygosity in the colonizing species. The results presented here show that the occurrence of a hexaploid was quite restricted in the sampling performed, and that the tetraploids also did not show a preferential pattern of distribution, so that these data did not allow establishing the same relationship between level of ploidy and latitude. On the other hand, in order to make such inferences about latitude more safely, it is necessary that a larger area of South America or even Brazil be investigated.

It should be pointed out that the *tall* type plants were found only in the Campos de Cima da Serra region, located in the extreme Northeast of RS. This area presents altitudes ranging from 900 to 1200 m and climate type *Cfb* (temperate

oceanic climate), according to the Köppen climatic classification (Moreno, 1961). This morphological plant type has 2*n* = 2*x* = 16, as well as the *medium* type. Maybe the differences in morphology are not the consequence of the chromosome number, but of the amount of DNA. If such differences are reflecting larger amount of DNA, they could be expressed either in the chromosome number or in their size. However, the meiotic analyses do not suggest differences in chromosome size. In this case, karyotypic analyses (made from mitotic cells) and flow cytometry analyses might help explain these issues.

Based on the current analyses, it is not yet possible to establish a concise identification of the morphological types from the cytogenetic data and morphological characterization.

The molecular data show that the accessions are well structured, forming distinct groups, but at the same time, the relationship between the types is not very clear. Initially it was expected that three main, cohesive groups would be formed, so that each would correspond to a distinct morphological type. Later, based on cytogenetic analyses, it was imagined that there would be a grouping according to chromosome number. However, the accessions of the *tall* type (ESC164, ESC170 and ESC198, all 2*x* = 16) were all in the same main cluster, but not on the same branch. ESC170 and ESC198 proved rather similar, while ESC164 was grouped with ESC176 (*medium* type, 2*x* e 4*x*). Recalling that these are accessions from different regions (São Francisco de Paula and Pantano Grande, respectively) it is difficult to imagine that a gene flow is taking place between the accessions, both via pollen, and through seeds. But this

similarity relationship does not necessarily imply a same origin, it only indicates that in both areas sampled, the individuals resemble each other genetically. Considering that accession ESC177 (*short* type) belongs to the same region as ESC176, it was expected that both would be grouped on a same branch. But, whereas ESC176 was grouped with ESC164, ESC177 was grouped with ESC197 (*short* type, 4*x*). The latter two accessions also belong to distinct areas, between which there is probably no allele exchange. However, the grouping between them suggests that access ESC177 may also be constituted by tetraploid individuals.

On the other hand, the two other accessions constituted by *medium* type individuals (ESC160 and ESC175, 2*x*) were grouped in the same subcluster, and there is a great similarity between the individuals of the two different accessions. Furthermore, accession ESC172 (probably 6*x*) presented greater similarity to this group of medium-sized accessions, than to the others in which polyploid 4*x* individuals were found. Considering these aspects, it is noted that the relationships are a lot more complex. The processes involved in this broad diversification appear to be involved in polyploidization, so that it could be verified that the areas of occurrence of this species may be constituted by more than one level of ploidy. It should be stressed that these cytogenetic, molecular and morphological data are new, and show the importance of maintaining these populations in the State of Rio Grande do Sul, Brazil, to preserve this variability and continuity in the evolutionary process of the species.

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Table 1. Sample sites of *Sisyrinchium micranthum* in southern Brazil.

Acession (Voucher)	Locality	Latitude (°S)	Longitude (°W)
ESC185	Caçapava do Sul	30°42'57.6''	53°30'17.3''
ESC205	Canoas	29°59'50.66''	51°11'03.69''
ESC180	Encruzilhada do sul	30°31'11.4''	52°41'41.7''
ESC182	Encruzilhada do sul	30°43'31.5''	52°37'33.5''
ESC175	Novo Hamburgo	29°43'47.67''	51°03'55.72''
ESC176	Pantano Grande	30°17'58.0''	52°21'57.7''
ESC177	Pantano Grande	30°17'58.0''	52°21'57.7''
ESC178	Pantano Grande	30°17'58.0''	52°21'57.7''
ESC184	Piratini	31°20'	52°44'
ESC189	Piratini	31°43'20.9''	52°53'30.2''
ESC196	Porto Alegre	30°04'13.88''	51°07'11.63''
ESC197	Porto Alegre	30°04'34.03''	51°07'30.58''
ESC164	São Francisco de Paula	29°26'01.8''	50°31'25.1''
ESC166	São Francisco de Paula	29°26'01.8''	50°31'25.1''
ESC170	São Francisco de Paula	29°23'19.30'	50°25'48.9''
ESC171	São Francisco de Paula	29°16'19.6''	50°25'48.91''
ESC198	São Francisco de Paula	29°27'00.5''	50°36'14.1''
ESC202	São Francisco de Paula	29°14'26.9''	50°16'07.7''
ESC160	Taquara	29°41'21.0''	50°49'0.03''
ESC161	Taquara	29°41'21.0''	50°49'0.03''
ESC172	Viamão	30°23'09.4''	51°01'07.2''
ESC173	Viamão	30°22'58.5''	51°01'02.3''
ESC174	Viamão	30°21'57.2''	51°01'45.9''
ESC195	Viamão	30°21'44.0''	51°01'13.0''
ESC208	Viamão	30°21'30.4''	51°02'48.5''

Table 2. Percent of meiotic irregularities in pollen mother cells, meiotic indexes and pollen fertility in southern Brazilian accessions.

Morphological type	Acessions	Chromosome number		Meiosis I				Meiosis II		Meiotic index		Pollen viability	
				Percent of irregularities in metaphase I		Percent of irregularities in anaphase and telophase I		Percent of irregularities in anaphase and telophase II					
		N[1]	*n*	N[1]	%	N[1]	%	N[1]	%	N[1]	%	N[1]	%
Medium light violet	ESC176	49 (2)	16	61 (2)	31.1	6 (2)	50.0	92 (2)	3.3	800 (4)	98.4	5 000 (10)	94.5
	ESC173	40 (1)	8	394 (1)	4.1	53 (1)	3.8	--	--	600 (3)	98.0	5 000 (10)	98.0
	ESC175	66 (3)	8	--	--	37 (1)	27.0	8 (1)	37.5	551 (3)	96.0	5 000 (10)	97.1
	ESC160	20 (1)	8	20 (1)	90.0	--	--	--	--	200 (1)	98.0	3 500 (7)	97.9
	ESC166	20 (1) 3 (1)	8 16	73 (1)	17.8	--	--	--	--	229 (2)	86.5	4 000 (8)	93.3
	ESC171	14 (1) 6 (1)	8 16	--	--	55 (1)	1.8	--	--	400 (2)	99.5	5 000 (10)	88.2
	ESC184	5 (2)	16	15 (1)	20.0	--	--	--	--	324 (2)	76.9	5 000 (10)	90.8
	ESC174	--	--	--	--	59 (1)	13.6	10 (1)	20.0	600 (3)	97.2	5 000 (10)	88.0
	ESC182	--	--	--	--	--	--	--	--	593 (3)	82.5	5 000 (10)	96.3
	ESC195	--	--	--	--	--	--	--	--	600 (3)	99.0	4 000 (8)	94.5
	ESC208	--	--	--	--	--	--	--	--	799 (4)	94.9	5 000 (10)	97.6

[1]N corresponds to the number of analyzed cells and the number of individuals is in parenthesis.

Table 2. Continued.

Morphological type	Accessions	Chromosome number		Meiosis I				Meiosis II		Meiotic index		Pollen viability	
				Percent of irregularities in metaphase I		Percent of irregularities in anaphase and telophase I		Percent of irregularities in anaphase and telophase II					
		N[1]	*n*	N[1]	%	N[1]	%	N[1]	%	N[1]	%	N[1]	%
Medium light violet	ESC180	--	--	--	--	--	--	--	--	--	--	5 000 (10)	93.4
	ESC188	--	--	--	--	--	--	--	--	--	--	5 000 (10)	77.1
	ESC189	--	--	--	--	--	--	--	--	--	--	5 000 (10)	95.0
	ESC196	--	--	--	--	--	--	--	--	--	--	5 000 (10)	95.7
	ESC197	--	--	--	--	--	--	--	--	--	--	5 000 (10)	96.7
	ESC198	--	--	--	--	--	--	--	--	--	--	5 000 (10)	97.5
	ESC205	--	--	--	--	--	--	--	--	--	--	5 000 (10)	96.6
	ESC185	--	--	--	--	--	--	--	--	--	--	5 000 (10)	97.4
	Total	**160 (7)** **63 (6)**	**8** **16**	**563 (6)**	**12.3**	**210 (6)**	**11.4**	**110 (4)**	**7.3**	**5 696 (30)**	**94.3**	**91 512 (183)**	**94.0**

[1]N corresponds to the number of analyzed cells and the number of individuals is in parenthesis.

Table 2. Continued.

Morphological type	Acessions	Chromosome number		Meiosis I				Meiosis II		Meiotic index		Pollen viability	
				Percent of irregularities in metaphase I		Percent of irregularities in anaphase and telophase I		Percent of irregularities in anaphase and telophase II					
		N[1]	2*n*	N[1]	%	N[1]	%	N[1]	%	N[1]	%	N[1]	%
Medium light yellow	ESC160	14 (1)	8	--	--	--	--	--	--	200 (1)	99.0	5 000 (10)	80.4
	ESC197	--	--	--	--	--	--	--	--	--	--	3 000 (6)	89.9
	ESC205	--	--	--	--	--	--	--	--	--	--	5 000 (10)	90.8
	Total	**14 (1)**	**8**					**--**	**--**	**200 (1)**	**99.0**	**13 000 (26)**	**89.7**
Medium pink	ESC160	28 (1)	8	127 (1)	14.2	96 (1)	2.1	--	--	200 (1)	99.5	4 000 (8)	97.5
	ESC178	--	--	--	--	--	--	--	--	--	--	1 000 (2)	63.5
	ESC189	--	--	--	--	--	--	--	--	--	--	5 500 (11)	90.9
	ESC205	--	--	--	--	--	--	--	--	--	--	5 000 (10)	92.4
	Total	**28 (1)**	**8**	**127 (1)**	**14.2**	**96 (1)**	**2.1**	**--**	**--**	**200 (1)**	**99.5**	**15 500 (31)**	**91.3**
Tall light violet	ESC164	55 (1)	8	105 (2)	2.9	--	--	--	--	200 (1)	100.0	4 500 (9)	81.4
	ESC170	45 (1)	8	3836 (4)	6.3	721 (2)	11.7	--	--	400 (2)	99.0	4 500 (9)	97.0
	ESC198	68 (3)	8	126 (2)	8.7	23 (1)	4.3	--	--	853 (5)	97.4	5 000 (10)	79.3
	ESC202	--	--	--	--	--	--	--	--	--	--	5 000 (10)	92.3
	Total	**196 (6)**	**8**	**4194 (9)**	**6.1**	**840 (4)**	**10.1**	--	--	**1 653 (8)**	**86.3**	**19 000 (38)**	**87.4**

[1]N corresponds to the number of analyzed cells and the number of individuals is in parenthesis.

Table 2. Continued.

Morphological type	Accessions	Chromosome number		Meiosis I				Meiosis II		Meiotic index		Pollen viability	
				Percent of irregularities in metaphase I		Percent of irregularities in anaphase and telophase I		Percent of irregularities in anaphase and telophase II					
		N[1]	2*n*	N[1]	%	N[1]	%	N[1]	%	N[1]	%	N[1]	%
Short light violet	ESC160	--	--	--	--	--	--	13 (1)	0.0	200 (1)	99.0	4400 (9)	93.6
	ESC172	--	--	--	--	--	--	--	--	1018 (6)	97.0	5000 (10)	97.8
	ESC177	--	--	--	--	--	--	--	--	125 (1)	96.8	3000 (6)	70.0
	ESC197	21 (1)	16	37 (1)	8.1	5 (1)	0.0	--	--	--	--	1000 (2)	97.5
	Total	**21 (1)**	**16**	**37 (1)**	**8.1**	**6 (1)**	**0.0**	**13 (1)**	**0.0**	**1343 (8)**	**97.2**	**13400 (27)**	**88.3**
Short light yellow	ESC161	--	--	62 (1)	3.2	--	--	--	--	476 (4)	98.5	2500 (5)	91.8
	ESC172	30 (1)	24	112 (1)	26.8	--	--	--	--	390 (4)	90.0	5000 (10)	97.4
	Total	**30 (1)**	**24**	**174 (2)**	**18.4**	**--**	**--**	**--**	**--**	**866 (8)**	**94.7**	**7500 (15)**	**95.5**

[1]N corresponds to the number of analyzed cells and the number of individuals is in parenthesis.

Table 3. Anther measurements for meiosis, tetrads and microspore analysis.

Morphological type	Cell division stage					
	Meiosis I and II		Tetrad		Microspore	
	N[1]	Average anther length (mm)	N[1]	Average anther length (mm)	N[1]	Average anther length[2] (mm)
Tall light violet	30 (10)	3.52	36 (12)	3.74	114 (38)	7.71[a]
Medium light violet	60 (20)	2.99	87 (30)	3.26	539 (181)	6.63[b]
Medium light yellow	3 (1)	3.53	3 (1)	4.10	81 (27)	6.53[bc]
Medium pink	6 (2)	2.33	3 (1)	3.27	96 (32)	6.13[d]
Short light violet	3 (1)	1.90	26 (8)	2.46	69 (23)	4.86[e]
Short light yellow	9 (3)	2.33	26 (9)	2.20	45 (15)	4.51[e]

[1]N corresponds to the number of analyzed cells and the number of individuals is in parenthesis.

[2] Values within columns with different letters differ at $P < 0.05$.

Table 4. Results of pollen longer axis (P), pollen shorter axis (E), area and ratio P/E, of the morphological types analyzed and their respective chromosome numbers (*n*).

Morphological type	Chromosome number (*n*)	N[1]	Pollen longer axis (P)	Pollen shorter axis (E)	Area	Ratio P/E
			Average[2] (µm)	Average[2] (µm)	Average[2] (µm^2)	Average
Short light yellow	24	285 (15)	36.35[a]	30.34[a]	3513.07[a]	1.1973
Medium pink	8	608 (32)	31.32[c]	27.32[b]	2763.18[bc]	1.1777
Short light violet	16	437 (23)	33.36[b]	26.95[b]	2863.90[b]	1.2423
Medium light violet	8 and 16	3414 (181)	31.35[c]	26.94[b]	2666.90[c]	1.1738
Tall light violet	8	722 (38)	29.71[d]	26.02[c]	2451.91[d]	1.1512
Medium light yellow	8	513 (27)	30.73[d]	25.19[d]	2447.11[e]	1.2246

[1]N corresponds to the number of analyzed cells and the number of individuals is in parenthesis.

[2] Values within columns with different letters differ at $P < 0.05$.

Table 5. ISSR primers used, total number of fragments scored for each primer and the size of the amplified fragments.

Primer	Sequence (5 '- 3')	N° of recorded loci	Size range (bp)
P2	$(GA)_8T$	13	400 - 1534
P3	$(CTC)_4RC$	21	250 - 1634
P4	$(CT)_8G$	9	480 - 1534
F3	$(AG)_8C$	17	350 - 1634
F4	$(GA)_8C$	17	260 - 1634
F11	$(GACA)_4$	16	550 - 1740
Total		93	

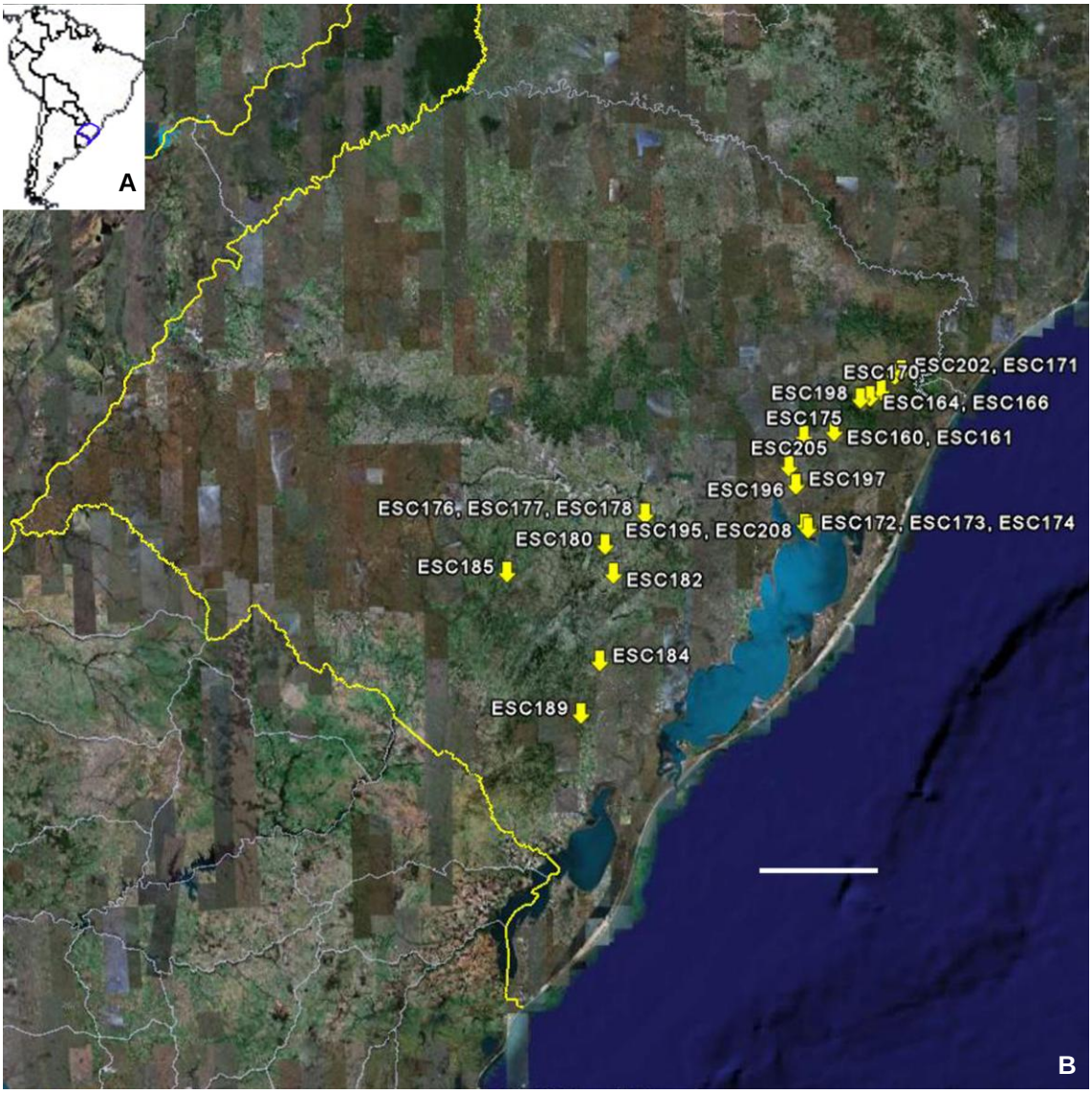

Fig. 1. (**A**) Map of South America; (**B**) Rio Grande do Sul state with the sampling sites. For accession names and coordinates see Table 1. Bar = 100 km.

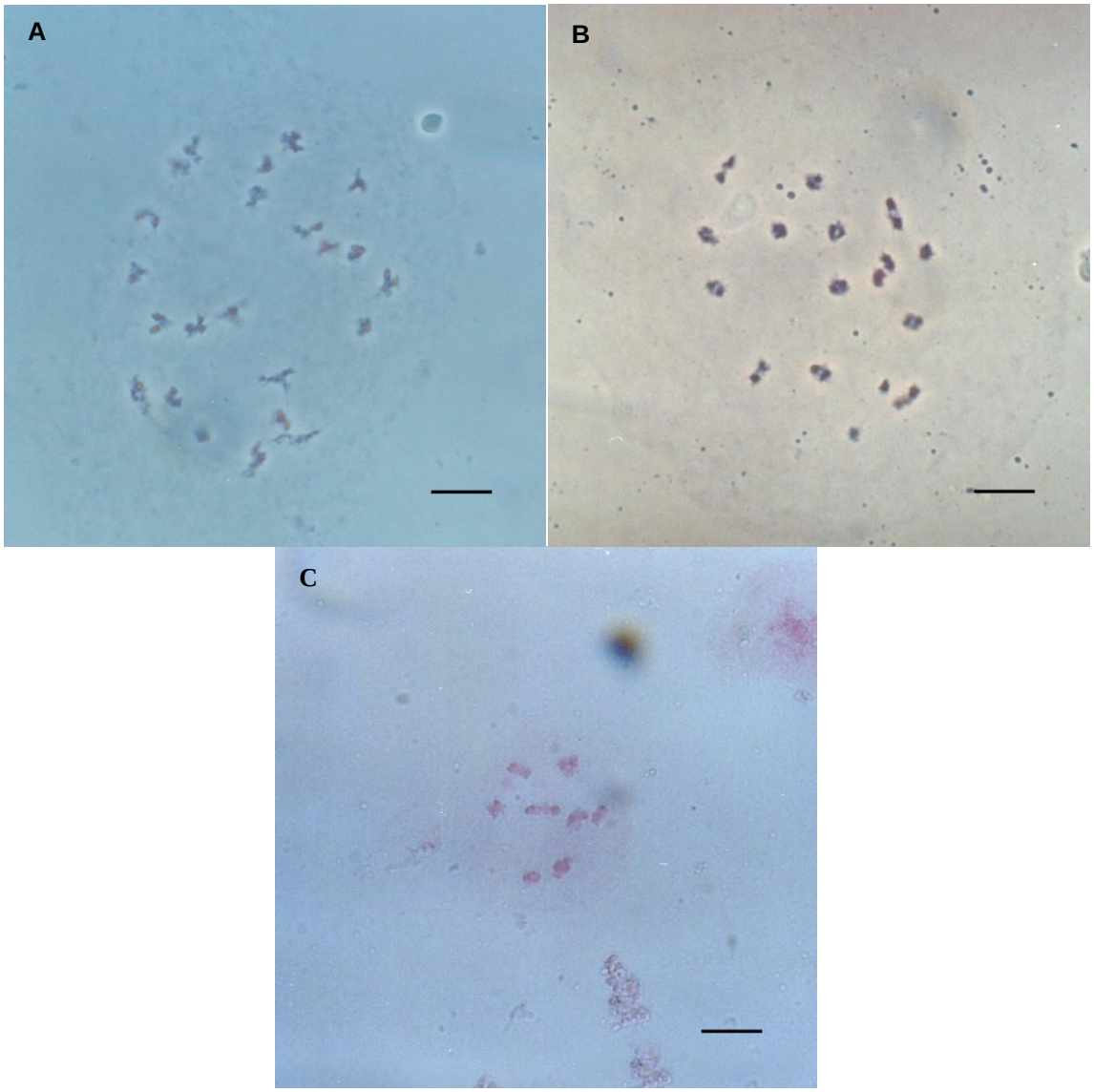

Fig. 2. Three cytotypes present in *Sisyrinchium micranthum* in Rio Grande do Sul, Brazil. Cells in diakinesis. (**A**) ESC172 (*Short* light yellow, *n* = 24); (**B**) ESC176 (*Medium* light violet, *n* = 16); (**C**) ESC164 (*Tall* light violet, *n* = 8). Bar = 10 µm.

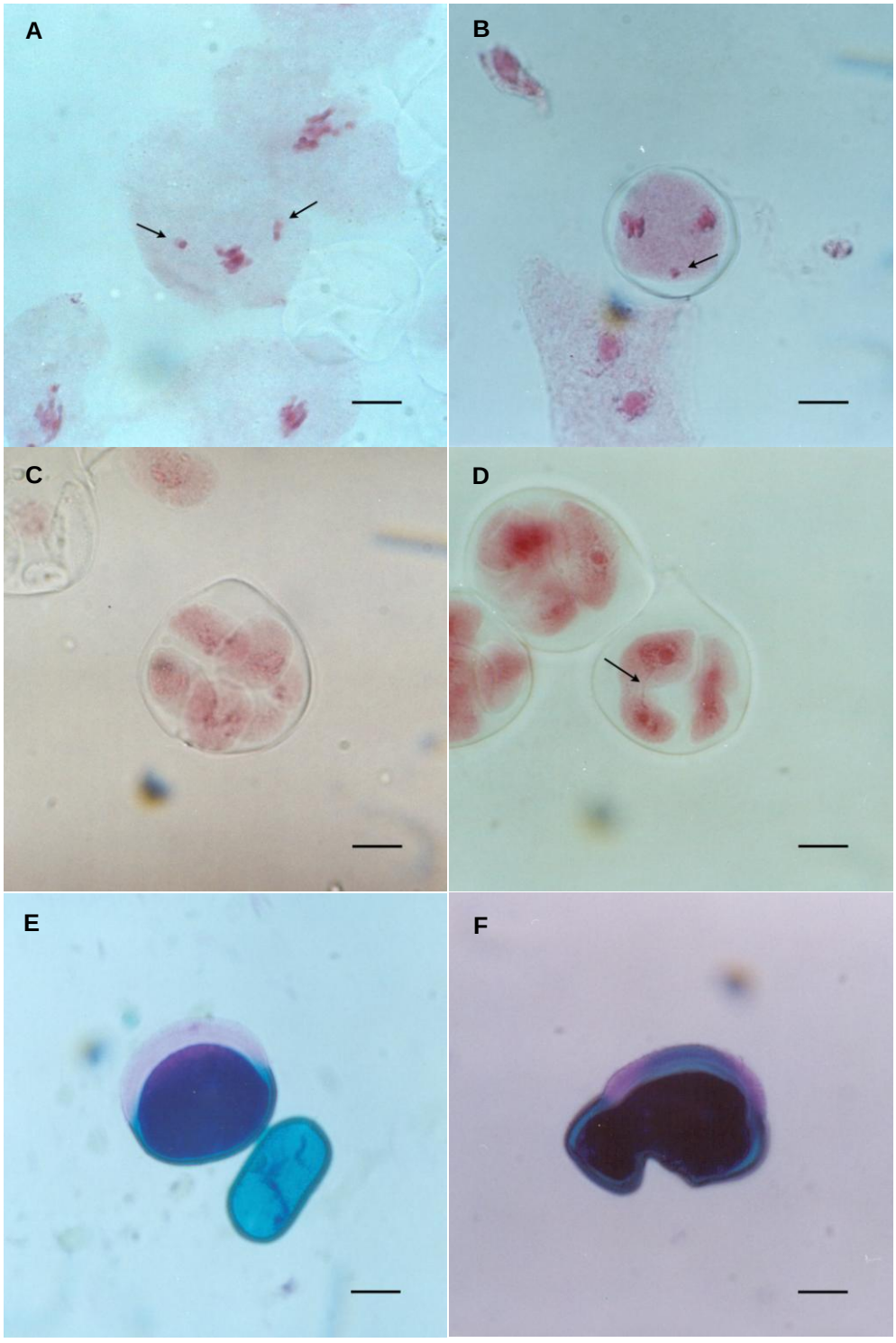

Fig. 3. Meiotic behavior in pollen mother cells. Some abnormalities: (**A**) Metaphase I with not aligned chromosomes; (**B**) Telophase I with laggard; (**C**) “Tetrad” with 6 cells; (**D**) Tetrad with chromosome bridge; (**E**) Viable (dark) and unavailable pollen grains (light); (**F**) Abnormal pollen grain. Bar = 10 µm.

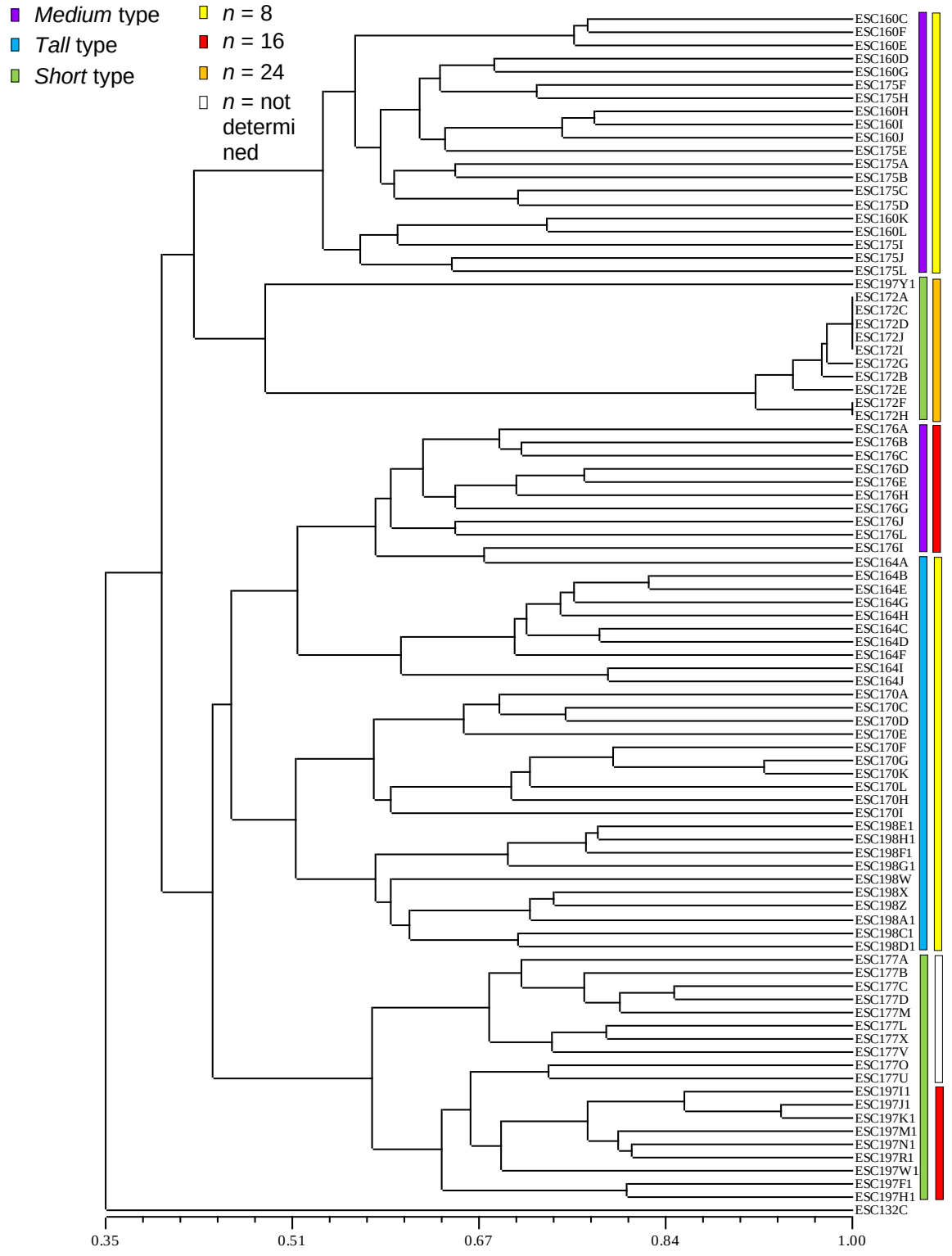

Fig. 4. Unweighted pair-group method arithmetic average (UPGMA) dendrogram based on Jaccard's index portraying genetic similarity among morphological types, accessions and individuals of *Sisyrinchium micranthum*

(*Short* type: ESC172, ESC177 and ESC197; *Medium* type: ESC160, ESC175 and ESC176; *Tall* type: ESC164, ESC170 and ESC198). For chromosome numbers see Table 2. A scale for genetic distance is provided at the bottom of the graph.

DISCUSSÃO

Os resultados citológicos e moleculares obtidos neste trabalho constituem dados inéditos e levantam uma série de questões a respeito da diversificação de *Sisyrinchium micranthum*.

Os números cromossômicos descritos na literatura para esta espécie são de 4*x* = 32 e 6*x* = 48, relativos a espécimes coletados no Hemisfério Norte, sendo o provável número cromossômico básico *x* = 8 (Goldblatt, 1982; Kenton and Heywood, 1984; Goldblatt and Takei, 1997).

As análises de contagem cromossômica permitiram verificar a ocorrência dos dois níveis de ploidia no Rio Grande do Sul, sendo que *n* = 24 foi encontrado dentro do Parque Estadual de Itapuã. Indivíduos diplóides, *n* = 8, foram descritos pela primeira vez para a espécie, sendo este nível de ploidia mais freqüente nas populações do Estado. Com relação à morfologia, era esperada uma relação entre o porte e o nível de ploidia dos indivíduos já que poliplóides, geralmente, apresentam em sua morfologia o efeito "*giga"*, o que resulta em flores, grãos de pólen e sementes maiores (Ramsey and Schemske, 2002). Contudo, quanto ao tamanho das plantas e às suas estruturas florais esse padrão não foi encontrado e, ao contrário do esperado, as plantas hexaplóides apresentaram porte menor do que as tetraplóides e diplóides. Apenas os grãos de pólen dos hexaplóides refletiram o efeito do nível de ploidia, sendo que estes apresentaram tamanho significativamente maior.

A variação morfológica existente para a espécie no Estado é bastante ampla, de forma que a extensa gradação de tipos morfológicos dificulta a categorização dos indivíduos. Isto se deve, provavelmente, porque em alguns casos, dentro de uma mesma população, foram encontrados mais de um nível de ploidia.

As análises relativas à variação genética populacional e ao número cromossômico realizadas para as populações no Parque Estadual de Itapuã sugerem que os acessos analisados dentro do Parque são uniformes quanto ao número cromossômico. Ou seja, não parece haver diferentes números cromossômicos dentro de cada uma das populações. As plantas de porte médio são, provavelmente, todas diplóides (n = 8), enquanto a população com porte menor é provavelmente hexaplóide, considerando-se os agrupamentos baseados nas análises estatísticas. As populações mostraram uma forte estruturação genética com pouco fluxo gênico entre as mesmas.

As análises moleculares para os morfotiipos permitiram verificar que os acessos foram constituídos quase que exclusivamente pelos indivíduos correspondentes aos sítios de coleta, mostrando-se bastante estruturados também. Mas o acesso ESC172, de número cromossômico hexaplóide, n = 24, destacou-se pelos altos níveis de similaridade entre os indivíduos em todas as análises.

Todos estes resultados evidenciam que a poliploidia tem sido um fator de extrema importância na diversificação desta espécie. A regularidade meiótica e a elevada viabilidade polínica poderiam sugerir uma origem por alopoliploidia. Porém, uma origem autopoliplóide não pode ser descartada uma

vez que se desconhece há quanto tempo estes poliplóides estão estabelecidos e o quanto os processos de reestruturação genômica e cromossômica podem estar influenciando no comportamento meiótico dos mesmos. Soltis and Soltis (1999), revisando vários aspectos importantes da poliploidia, salientam que a formação recorrente somada à reorganização do genoma e dos cromossomos tem implicações evolutivas e genéticas importantes, pois constituem fontes adicionais de diversidade genética. Considerando-se que se estima que 60% das espécies de Iridaceae do Hemisfério Norte são poliplóides e que nas Américas do Sul e Central, praticamente todas as espécies já investigadas da tribo Tigridieae são tetra e hexaplóides, a poliploidia evidentemente constitui um fator importante na diversificação desta família (Goldblatt and Takei, 1997).

No entanto, pouco se sabe a respeito da biologia reprodutiva do gênero *Sisyrinchium,* e como a poliploidia tem atuado modificando a fenologia e os caracteres reprodutivos nas várias espécies que compõem o gênero, principalmente, as espécies brasileiras. Da mesma forma, as alterações associadas ao comportamento dos polinizadores são desconhecidas. Para algumas espécies de *Sisyrinchium* da América do Norte já foram descritas modificações na maturação dos órgãos reprodutivos florais, relacionadas com o nível de ploidia das plantas. De forma que espécies protândricas apresentaram redução no intervalo de amadurecimento das partes reprodutivas conforme um aumento no nível de ploidia das espécies estudadas, facilitando a autofertilização nas espécies autocompatíveis (Henderson, 1976). Além disso, neste mesmo estudo, a autocompatibilidade também foi mais freqüente nas espécies com altos níveis de ploidia.

Geralmente, espera-se que as plantas poliplóides apresentem taxas maiores de autofecundação do que seus progenitores diplóides (Stebbins, 1950). Nas espécies com mecanismos controlados geneticamente contra a autofecundação, a poliploidia poderia ser facilitada com a quebra desse sistema de incompatibilidade. No entanto, Mable (2004) acessando uma ampla base de dados a respeito dos números cromossômicos, níveis de ploidia e sistema de compatibilidade de angiospermas, verificou que inexistem evidências de associação entre poliploidia e autocompatibilidade.

Sisyrinchium micranthum foi descrito por Truylio *et al.* (2002) como uma espécie de fecundação cruzada, auto-incompatível com diferenças no período de amadurecimento das partes reprodutivas (protoginia). Considerando-se que as plantas diplóides são as mais freqüentes no Estado, é provável que os indivíduos avaliados pelos pesquisadores neste estudo sejam também diplóides. Desta forma, os altos índices de similaridade encontrados para a população hexaplóide presente no PEI e os altos índices de diversidade nas outras populações podem indicar que os indivíduos hexaplóides sejam de autofecundação, enquanto os de nível de ploidia inferior seriam de fecundação cruzada.

Além disso, em relação à população ESC172 (6*x*), pode-se imaginar uma série de cenários que expliquem sua baixa diversidade genética: reprodução assexual, autofecundação, efeito fundador, alterações nas interações planta-polinizador, entre outros. Se estes têm influenciado independentemente ou concomitantemente a diferenciação desta população, ainda é uma incógnita.

A poliploidia constitui um mecanismo importante na especiação das plantas e no caso de *Sisyrinchium micranthum* é evidente seu papel para a diversificação desta espécie no Estado. A integração de diferentes ferramentas para avaliar e caracterizar essa variação foi de extrema importância e revelou a necessidade de cada vez mais buscar-se expandir os estudos com novas investigações e abordagens para compreensão dos processos evolutivos envolvidos como um todo.

Em vista de tantas questões a serem esclarecidas, as perspectivas futuras são de amostrar uma área maior de distribuição da espécie no Brasil e manter as abordagens citogenéticas e moleculares. Contudo serão empregadas as análises de citogenética molecular como FISH e GISH e marcadores moleculares de herança codominante do tipo microssatélites. Durante o período de mestrado, foram desenvolvidas bibliotecas enriquecidas de microssatélites, por nosso grupo de pesquisa e colaboradores, para *Sisyrinchium micranthum* e outras duas espécies que ocorrem no sul do Brasil, *S. vaginatum* e *S. palmifolium*. O desenvolvimento de marcadores moleculares do tipo microssatélites viabilizará análises populacionais adicionais, como estimativas de fluxo gênico e taxas de cruzamento. Considerando-se que este trabalho está agregado a um projeto maior intitulado “Biologia e evolução das espécies brasileiras de *Sisyrinchium* L. (Iridaceae)” no qual estão envolvidos pesquisadores dos departamentos de Genética e Botânica da UFRGS e da Universidade de Paris XI (Orsay, França), abordando diferentes áreas do conhecimento, considerações importantes a respeito da ecologia e biologia

reprodutiva serão agregadas e poderão gerar um panorama mais claro a respeito da evolução da espécie.

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