



Population genetic structure of *Sisyrinchium micranthum* Cav. (Iridaceae) in Itapuã State Park, Southern Brazil

Luana Olinda Tacuatiá¹, Lilian Eggers², Eliane Kaltchuk-Santos¹ and Tatiana T. Souza-Chies^{1,2}

¹Instituto de Biociências, Departamento de Genética, Programa de Pós-Graduação em Genética e Biologia Molecular, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil.

²Instituto de Biociências, Departamento de Botânica, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil.

Abstract

Sisyrinchium micranthum Cav. is a member of the family Iridaceae, which is distributed over the American continent. In Brazil, this species is found, not only in disturbed areas and coastal regions, but is also very common in urban centers, such as public parks, during the spring. Chromosome counts for North American specimens are $2n = 32$ and $2n = 48$, whereas in southern Brazil, there is a polyploidy series with three chromosome numbers, $2n = 16$, $2n = 32$, and $2n = 48$. Population analyses using DNA molecular markers are inexistent for this species, in spite of its wide distribution and morphological variation. To study the genetic population structure of *S. micranthum*, five natural populations were accessed in a conservation park within the Atlantic Rain Forest Biome in southern Brazil. Here, the chromosome numbers $2n = 16$ and $2n = 48$ had already been described. Molecular analysis showed that the populations are highly structured with low gene flow among them. The population with $2n = 48$ was genetically less variable than and distinct from the other populations. Population genetics in relation to cytogenetic data provided new insights regarding the genetic diversification and mating system of *S. micranthum*.

Key words: Iridaceae, ISSR-PCR, mating system, population genetics, *Sisyrinchium micranthum*.

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Introduction

Sisyrinchium micranthum Cav. (Iridaceae) is an herb species with violet, yellow, or pink flowers, violet being the most common color. This species produces floral oil in trichomatic structures called elaiophores, as a reward to pollinators (Cocucci and Vogel, 2001; Truylio *et al.*, 2002). *S. micranthum* is distributed in Americas (Johnston, 1938; Goldblatt, 2003), from south Argentina to Mexico. In Brazil, it is usually encountered in disturbed areas, and during the spring, it is commonly to be found flowering in urban centers, such as public parks. In south Brazil, this herb shows remarkable morphological variation, and different morphological categories (CI, CII, and CIII) have been adopted to classify plants based on morphological features, such as the number of internodes of the flowering stem, as well as the lengths of the flowering stem, the inferior internode, the peduncle, the outer and inner spathes and the staminal column (Tacuatiá LO, Flores AM, Souza-Chies

TT, Eggers L, Siljak-Yakovlev S, Kaltchuk-Santos E, submitted).

Iridaceae is represented by around 65 to 75 genera, with over 2030 species all told (Goldblatt *et al.*, 2008). Certain genera have been studied extensively, due to their economic importance as ornamental plants, food items and spices. Little is known, however, regarding most of those devoid of economic value, such as *Sisyrinchium* L. species. This genus is represented by approximately 140 species in America (Goldblatt *et al.*, 2008). Data on the biology, cytogenetics, and leaf anatomy of *Sisyrinchium* species 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), although little is known about most of those from South America, especially from Brazil.

Iridaceae species, including *Iris* L., *Moraea* Mill., *Crocus* L., *Gladiolus* L. and the genus *Sisyrinchium*, have extensive polyploid series (Ingram, 1968; Henderson, 1976; Cholewa and Henderson, 1984; Goldblatt *et al.*, 1984; Kenton and Heywood, 1984; Kenton *et al.*, 1986; Rudall *et al.*, 1986; Goldblatt and Takei, 1997). Polyploidy, reported in more than 70% of *Sisyrinchium* species

Send correspondence to Luana Olinda Tacuatiá. Instituto de Biociências, Departamento de Genética, Programa de Pós-Graduação em Genética e Biologia Molecular, Universidade Federal do Rio Grande do Sul, Avenida Bento Gonçalves 9500, Prédio 43312, Caixa Postal 15053, 91501-970 Porto Alegre, RS, Brazil. E-mail: tacuatiata@yahoo.com.br.

(Goldblatt and Takei, 1997), also appears to be related to the complex diversification of *S. micranthum* (Tacuatiá LO, Flores AM, Souza-Chies TT, Eggers L, Siljak-Yakovlev S, Kaltchuk-Santos E, submitted). Goldblatt (1982) described the chromosome number for introduced specimens collected in Texas as $2n = 32$, whereas native plants from Colombia (Kenton and Heywood, 1984) and Nicaragua (Goldblatt and Takei, 1997) presented $2n = 48$. In south Brazil, three cytotypes ($2x$, $4x$ and $6x$) have recently been recorded for *S. micranthum*, diploidy ($2n = 16$) being the most common (Tacuatiá LO, Flores AM, Souza-Chies TT, Eggers L, Siljak-Yakovlev S, Kaltchuk-Santos E, submitted). The allopolyploid origin of many cytotypes in the genus *Sisyrinchium* is proposed by Kenton *et al.* (1986), although consistent data are not available.

The population genetics of Iridaceae species have been intensively investigated (Burke and Arnold, 1999; Burke *et al.*, 2000; Hannan and Orick, 2000; Wilson *et al.*, 2000; Karst and Wilson, 2002; Wróblewska *et al.*, 2003; Caiola *et al.*, 2004; Meerow *et al.*, 2005, 2007; Marco *et al.*, 2009). Even so, and despite its wide distribution and interesting morphological and genetic features, *S. micranthum* has been neglected. The Itapuã State Park (Parque Estadual de Itapuã-PEI), where several populations of different morphological categories have been observed, is a state conservation area, dedicated to preserving the remaining original vegetation and impressive ecosystem diversity of the Atlantic Rain Forest Biome (Fonseca *et al.*, 2004).

The present study aims to investigate genetic variability within and among these populations by means of inter-simple sequence repeat (ISSR) markers.

Material and Methods

Population sampling

In 2005, five populations of *S. micranthum* were sampled in the PEI located in the municipality of Viamão, approximately 57 km from Porto Alegre, Rio Grande do Sul (RS), Brazil (Figure 1). The study sites consisted of a bank site, denominated Guaíba Lagoon, and sites comprising hills with granitic outcrops, viz., Praia de Fora, Pedra da

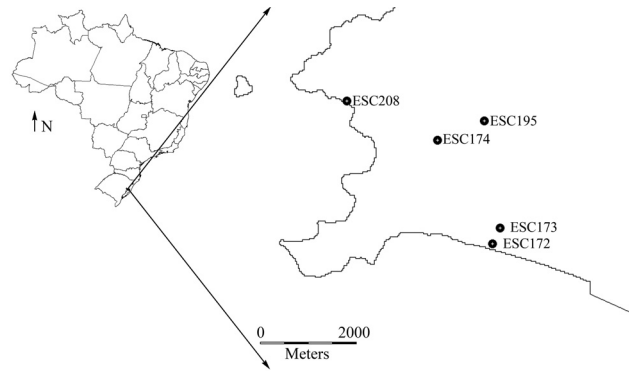


Figure 1 - Itapuã State Park (Parque Estadual de Itapuã-PEI), RS, Brazil and the five populations of *Sisyrinchium micranthum* studied: ESC172, ESC173, ESC174, ESC195, and ESC208.

Visão, Pedra da Grota and Praia da Pedreira. Four populations with light violet flowers and one with two flower colors, light violet and light yellow, were collected. Collection sites, coordinates, numbers of collected individuals, and flower colors appear in Table 1. Voucher specimens were deposited in the ICN Herbarium, Instituto de Biociências, Universidade Federal do Rio Grande do Sul.

DNA isolation and ISSR-PCR amplification

DNA sample extraction was based on the method of Doyle and Doyle (1987) with certain modifications. A set of twelve ISSR primers was tested, and six that generated good patterns with a representative sample group were further used for DNA amplification of all the populations. PCR was carried out in 25- μ L reactions using (depending on the primer): 4% DMSO, 1x buffer, 4.6-5.0 mM $MgCl_2$, 0.48-0.8 mM dNTP mixture (Invitrogen, São Paulo, Brazil), 0.4-0.6 mM of each primer, 1 U *Taq* DNA polymerase (CenBiot, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil), and 10 ng of genomic DNA. The thermal cycling program for amplification consisted of initial denaturation at 92 °C for 5 min, followed by 35 cycles of denaturation at 94 °C for 1 min, annealing at 42-45 °C (Table 2) for 1 min, extension at 72 °C for 2 min 30 s, and a final extension step at 72 °C for 5 min. PCR products were analyzed on 1.5% agarose gels and stained with GelRed (Amicon Corp., Lexington, MA).

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

Population (Voucher)	Sampling site	Latitude (°S)	Longitude (°W)	No. of individuals	Flower color
ESC172	Praia de Fora	30°23'09.4"	51°01'07.2"	9	light violet
				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				114	

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

Primer	Sequence (5'-3')	Annealing temperature (°C)	No. of recorded markers	Size range (bp)
P ₂	(GA) ₈ T	45	13	400-1800
P ₃	(CTC) ₄ RC	45	16	450-1634
P ₄	(CT) ₈ G	42	9	480-1534
F ₃	(AG) ₈ C	45	14	350-1434
F ₄	(GA) ₈ C	44	14	325-1684
F ₁₁	(GACA) ₄	43	13	550-1634
Total			80	

Statistical analyses

Bands were scored as binary characters based on their presence (1) or absence (0) and an unbiased genetic distance matrix (Nei, 1978) was generated by TFPGA version 1.3 (Tools for Population Genetic Analyses; Miller, 1997) to construct an unweighted pair-group method arithmetic average (UPGMA) topology, which computed 1000 permutations and estimated the confidence limits of the dendrogram. Marker frequencies were estimated based on the Lynch and Milligan (1994) Taylor expansion estimate. One pairwise difference matrix generated by ARLEQUIN version 3.11 (Excoffier *et al.*, 2005) with 1000 permutations, was used with MEGA version 4.0 (Tamura *et al.*, 2007) to produce an UPGMA dendrogram.

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

A hierarchical analysis of molecular variance (AMOVA; Excoffier *et al.*, 1992) was obtained with ARLEQUIN version 3.11 to determine the variance components and their significance levels.

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

et al., 2002). Estimates of genetic diversity (hs; defined as average panmictic heterozygosity) within each population were also calculated.

STRUCTURE version 2.3.1 (Falush *et al.*, 2007) was employed to obtain additional insights regarding gene flow and population subdivision. The most likely number of populations (k) was estimated under the admixture model and correlated allele frequencies, with no prior information on population origin. The program was run for 10,000 iterations, after a burn-in length of 10,000 iterations, to test population subdivision from $k = 1$ to $k = 10$, and thereby check for any possible subdivision. Twenty runs were carried out for each k , to quantify variation in likelihood, as a means of checking whether different runs could produce different likelihood values. Individual and average admixture proportions (Q) for each population in each genetic cluster found by the program, were recorded for the model. As an aid in identifying the number of clusters of individuals (k), the results generated by STRUCTURE were subsequently analyzed by way of the STRUCTURE HARVESTER version 0.6.7 (Earl, 2011), according to the method of Evanno *et al.* (2005). This method uses an *ad hoc* statistic k , based on the rate of change in the log probability of data between successive k values (see Evanno *et al.*, 2005, for a better explanation).

Results

The six primers produced 80 computable bands, of which 98.75% were polymorphic. The ISSR fragments generated an average of 13.2 bands per primer. The size of the amplified products ranged from 325 to 1800 bp (Table 2), the percentages of polymorphic loci from 43.8% (ESC172) to 78.8% (ESC195; Table 3), and genetic diversity indices within each population from 0.19 to 0.25 (results from Bayesian analysis in HICKORY, full model; Table 3).

Both, the UPGMA produced by TFPGA (Figure 2) based on the Nei's unbiased genetic distance matrix, and the UPGMA dendrogram (not shown) generated by MEGA based on the Φ_{ST} pairwise difference matrix, presented two main clusters, one comprising only the ESC172 population and the second two subclusters (bootstrap value about 98%), the first subcluster consisting of the ESC173 and ESC195 populations, and the second of the ESC174 and

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

Population	Percentage of polymorphic loci	Genetic diversity (hs)
ESC172	43.8	0.19
ESC173	72.5	0.25
ESC174	75.0	0.25
ESC195	78.8	0.25
ESC208	75.0	0.24

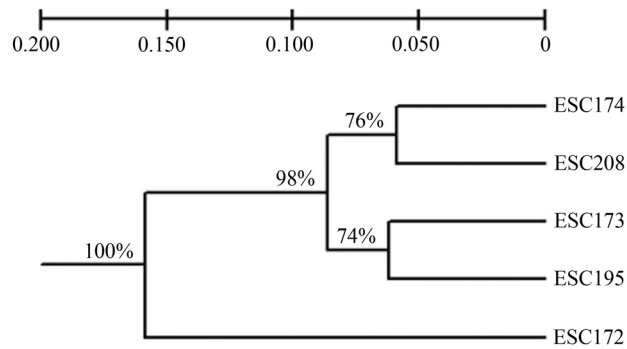


Figure 2 - Unweighted pair-group method arithmetic average (UPGMA) based on Nei (1978) genetic distance (including bootstrap support values in percentages) of populations of *Sisyrinchium micranthum* in the Itapuã State Park (Parque Estadual de Itapuã-PEI): ESC174, ESC208, ESC173, and ESC195, and ESC172. A scale of genetic distances is provided at the top of the graph.

ESC208 populations (74% and 76% of bootstrap values, respectively).

AMOVA generated Φ statistics, one analogous to Wright's F statistics. The analysis revealed that approximately 33% ($\Phi_{ST} = 0.3372$, $p < 0.001$) of genetic diversity could be attributed to divergence among populations, and 66% between individuals within a population ($\Phi_{IS} = 0.6628$, $p < 0.001$).

The Mantel test showed no significant correlation between geographic and genetic distances ($r = 0.2663$, $p = 0.2204$).

Data obtained through Bayesian genetic-structure analyses of *S. micranthum* furnished additional insights into population differentiation and gene flow. According to population analysis performed with HICKORY, the best model, *i.e.*, that which presented the lowest DIC (DIC = 1424.0), was the full model, with $\theta^B = 0.49$ and $f = 0.65$. The second best was $f = 0$ (DIC = 1468.4), with $\theta^B = 0.43$. Although in both cases, θ^B values were higher than the Φ_{ST} presented by AMOVA, all the F_{ST} analogues detected outstanding population differentiation. However, the values of inbreeding coefficients derived from analyses were very similar, $\Phi_{IS} = 0.66$ (ARLEQUIN, AMOVA) and

$f = 0.65$ (HICKORY, full model). The f -free model presented $f = 0.50$ and $\theta^B = 0.49$, the same result for θ^B presented by the full model, and DIC = 1582.0. The worst model of all was $\theta^B = 0$, resulting in $f = 0.91$, and DIC = 3853.5.

The $k = 3$ model was the most adequate for elucidating clustering (Figure 3). The decision was made based on statistic k , in so far as the uppermost peak of its modal value corresponded to the number of clusters detected by the software (Figure 4). The clustering into three groups corresponded exactly to the UPGMA produced by TFPGA (Figure 2); populations ESC174 and ESC208, and, ESC173 and ESC195 grouped together, and accession ESC172 represented apart in a third cluster (Figure 3).

Discussion

In spite of belonging to such a diversified family as Iridaceae, *Sisyrinchium micranthum* has never undergone population analysis using DNA molecular markers, whereby the importance of population genetics, not only in the specific case of diversity analysis, but also in bringing together other primary studies of this species. Based on Nei (1978) genetic distance, it was possible to cluster the differ-

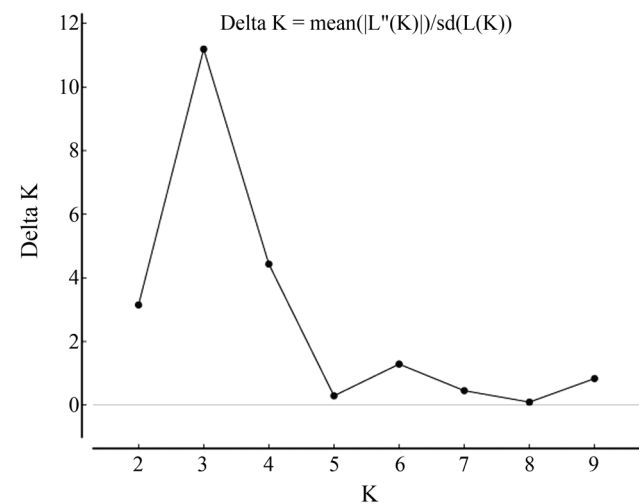


Figure 4 - Magnitude of the modal value of k as a function of k (mean \pm SD over 20 replicates).

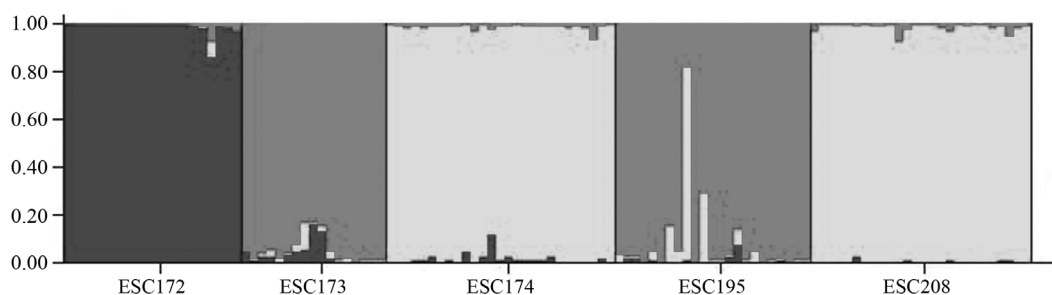


Figure 3 - Bayesian admixture proportions (Q) of individual plants of *Sisyrinchium micranthum* for $k = 3$. Each individual is represented by a single vertical line broken into k colored segments, with lengths proportional to each of the k -inferred clusters. Accessions from Itapuã State Park (Parque Estadual de Itapuã-PEI): ESC172, ESC173, ESC174, ESC195, and ESC208. The most likely number of populations (k) was estimated with the admixture model and correlated allele frequencies, with no prior information regarding population origin.

ent accessions into well-supported branches (Figure 2). The populations ESC173, ESC174, ESC195, and ESC208 were grouped into one of the two main clusters, and ESC172 into the other. Through previous cytogenetic analysis, the haploid chromosome numbers of three proved to be $n = 8$ (ESC173 and ESC208) and $n = 24$ (ESC172), corresponding to the somatic numbers $2n = 16$ (diploid) and $2n = 48$ (hexaploid), respectively (Tacuatiá LO, Flores AM, Souza-Chies TT, Eggers L, Siljak-Yakovlev S, Kaltchuk-Santos E, submitted). Since ESC195 and ESC174 clustered with ESC173 and ESC208, respectively, it is possible that both can be considered diploids.

The data obtained showed that the highly structured sites ($\Phi_{ST} = 0.3372$, $\theta^B = 0.49$) corresponded to different populations. Variance within populations, besides elucidating about 65%-66% ($\Phi_{IS} = 0.66$; and $f = 0.65$) of the total variation, corresponded well with the genetic structure of outcrossing plants. In general, there is more overall genetic variation and less differentiation among populations of outcrossing plants than in selfing plants (Hamrick and Godt, 1996). Truylio *et al.* (2002), when studying flower biology of *S. micranthum* in São Francisco de Paula, RS, in south Brazil, found that this species is protogynous, *i.e.*, female flower receptivity begins 6 to 7 h before male maturation. Moreover, in the same study, controlled pollination experiments indicated self-incompatibility. The pollinators were oil-bees from the family Apidae, of the tribe Tapinotaspini (Cocucci and Vogel, 2001; Truylio *et al.*, 2002), which usually nest close to foraging areas. However, syrphids and small pollen-collecting bees have already been seen visiting the flowers of *S. micranthum* (Truylio *et al.*, 2002; Freitas and Sazima, 2006). Although it is not clear how the plants investigated by Truylio *et al.* (2002) should be classified, as regards the morphological categories of *S. micranthum* adopted here, the low interpopulation gene flow verified in the present study might be related to pollinator behavior.

It has already been shown (Holtsford and Ellstrand, 1989; Martín *et al.*, 1997) that among herb species there is a correlation between high levels of intrapopulation variability and the breeding system. When using isozymes, Holtsford and Ellstrand (1989) found that, in the annual herb *Clarkia tembloriensis* Vasek (Onagraceae), the breeding system exerts a strong influence upon the distribution of genetic variation, both within and among populations. In this case, outcrossing populations had more total genetic variation and lower levels of differentiation among populations than the group of selfing plants. *Erodium paularense* Fern. Gonz. & Izco (Geraniaceae) is an outbreeding species, endemic to Spain. Population genetics studies showed that in this species, about 80% of all genetic diversity can be attributed to intrapopulation variation, thus consistent with the population structure of allogamous plants as a whole (Martín *et al.*, 1997).

It is important to note that in the ESC172 population, the percentage of polymorphic loci was lower, it was clustered separately from the other populations (Figure 2), in most individuals there was no admixture of alleles with those of the other populations (Figure 3), and the genetic diversity index was the lowest (Table 3). Thus, as there was no correlation between geographic and genetic distances; differentiation among collection sites remained enigmatic.

Since hexaploid plants (*e.g.*, ESC172) belong to the CI morphological type (reduced plant-size and anther-height, and larger pollen grains), according to the classification adopted for *S. micranthum* in south Brazil (Tacuatiá LO, Flores AM, Souza-Chies TT, Eggers L, Siljak-Yakovlev S, Kaltchuk-Santos E, submitted), the isolated clustering of the ESC172 population in the dendrogram might be related to the different chromosome numbers, thereby possibly associating polyploidy to this populational divergence.

These aspects raise questions as to whether the breeding system could be somehow related to genetic differentiation between ESC172 and the other populations analyzed, and polyploidy. Henderson (1976), when studying *Sisyrinchium* species from the Northern Hemisphere, reported a correlation between breeding system and ploidy level. Hand-selfing procedures showed that tetraploids were self-incompatible, whereas most of the higher polyploids were self-fertile. Furthermore, anthesis observations indicated that protandry (maturation of the anthers before the stigma) often occurred in blue-eyed grasses, thereupon inducing outcrossing, even in self-compatible plants. However, a higher ploidy level was associated with a shorter protandrous state, due to a decrease in the time interval between anther and stigma maturation according to an increase in ploidy level. Thus, while tetraploids were protandrous, most octoploids and dodecaploids presented a short or even no maturation time interval, thereby inducing higher levels of self-compatibility and self-pollination in plants with higher polyploidy.

Another interesting example is *S. bermudiana*, a species that occurs over a wide area of North America. This plant is self-fertile and presents a large range of chromosome numbers ($2n = 32, 64, 96$; Kenton *et al.*, 1986; Ingram, 1968). Its flowers are protandrous, since the anthers dehisce before the flower opens, whereas the stigma matures after opening. Furthermore, the length of the filament column is variable. Consequently, self-pollination is highly probable when the anther is at the same level as the style. Even so, outcrossing may occur when the filament column is shorter than the style (Ingram, 1968). Although, in the case of *S. bermudiana*, the relationship between ploidy level and self-pollination has not been investigated, from a previous study of *Sisyrinchium* (Henderson, 1976), it appears that the variation in length of the filament column in *S. bermudiana* could be related to the variation in chromosome number.

Based on the low values of genetic diversity presented by the polyploid accession in the PEI (ESC172), and considering that polyploidy, as mentioned above, may result in changes in the breeding system, this low genetic variation could arise from selfing. Thus, the populations, ESC173, ESC174, ESC195, and ESC208 are presumably diploids, self-incompatible and mainly outcrossing, according to the reported literature and the high estimated genetic diversity. On the other hand, since ESC172 is a polyploid population and genetically less variable, this population is possibly composed of self-compatible and self-fertile individuals.

However, the protogynous condition remains to be considered. As in the other *Sisyrinchium* species reported, the polyploidy process at ESC172 population could have been instrumental in breaking down this characteristic and/or self-incompatibility. Thus, as outcrossing would no longer be favored, self-pollination could occur.

As five specimens of ESC172 (with light-violet-colored flowers) presented the same band profile, four were excluded from statistical analyses. This pattern suggests that they are the result of self-fertilization or may even be clones. Thus, asexual reproduction may be contributing to the maintenance of this population. This is the first indication that reproduction in this species may not be strictly sexual. Thus, if reproduction is also vegetative, how would this fit into the mating system? On studying the relationship between mating system and life history (annual/perennial) in *Solanum* L. (Solanaceae), Vallejo-Marín and O'Brien (2007) found self-incompatibility and clonality to be strongly correlated. In their study, all of the self-incompatible plants were clonal and all strict annuals were self-compatible. Even so, in *Decodon verticillatus* (L.) Elliott (Lythraceae), clonality potentially furthers the increase of selfed offspring by way of geitonogamy (selfing through pollen transfer between flowers on the same plant; Eckert, 2000). Thus, the low genetic diversity in ESC172 individuals may possibly arise from self-fertilization alone, or as a consequence of both clonality and selfing. The connection of life-history with these aspects is unclear, as *S. micranthum* is usually cited as annual (Johnston, 1938; Innes, 1985; Goldblatt, 2003). Nonetheless, Parent (1987) reported specimens in northwest Spain that lived for more than one year, or at least survived the winter.

Even though whether something similar is occurring or has already occurred, and for how long, in *S. micranthum* in the PEI is unknown, this comprises an interesting mechanism for population maintenance in cases of a pre- or post-zygotic barrier between hexaploids and diploids at the beginning of colonization. Thus, the lower level of differentiation among individuals in the ESC172 population may be a consequence of initial population formation through the combination of a few polyploid specimens and the mating system.

Additional issues have emerged from the data regarding diversification and reproductive and pollination biology in this plant species. In order to adequately address the remaining questions, the need arises for alternative approaches to elucidate the mechanisms involved in its diversity, as well as a better understanding of its biology as a whole.

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