Isolation and characterization of microsatellite loci in *Sisyrinchium* (Iridaceae) and cross amplification in other genera

R.B. Miz[1], L.O. Tacuatiá[1], F.W. Cidade[2], A.P. de Souza[2,3], F. Bered[1], L. Eggers[4] and T.T. de Souza-Chies[1,4]

[1]Programa de Pós-Graduação em Genética e Biologia Molecular, Instituto de Biociências, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brasil
[2]Centro de Biologia Molecular e Engenharia Genética, Universidade Estadual de Campinas, Campinas, SP, Brasil
[3]Departamento de Biologia Vegetal, Instituto de Biologia, Universidade Estadual de Campinas, Campinas, SP, Brasil
[4]Departamento de Botânica, Instituto de Biociências, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brasil

Corresponding author: T.T. de Souza-Chies
E-mail: tatiana.chies@ufrgs.br

Genet. Mol. Res. 15 (3): gmr.15038474
Received January 20, 2016
Accepted June 23, 2016
Published September 16, 2016
DOI http://dx.doi.org/10.4238/gmr.15038474

ABSTRACT. Recent phylogenetic studies on *Sisyrinchium* strongly suggest that species classified in section *Hydastylus* and section *Viperella* belong to a single group of plants in recent adaptive radiation (Clade IV). These species neither present clear morphological differentiation among them nor show clear identification using DNA barcode markers. Thus, the main goal of this study was to develop a set of polymorphic microsatellite markers compatible for representative

species of both sections to ensure variability that could be revealed by SSR markers. Therefore, microsatellite primers were isolated and characterized for *Sisyrinchium palmifolium* and *S. marchioides*. In addition, transferability of the developed primers was tested in Iridoideae, primarily in closely related species of *Sisyrinchium*. Sixteen microsatellite loci were developed from enriched genomic libraries, of which ten were polymorphic. G_{ST} values indicated higher differentiation among subpopulations of *S. palmifolium* than those from *S. marchioides*. Major transferability was obtained using primers isolated from *S. marchioides*. All primers exhibited higher rates of cross-amplification for species belonging to Clade IV of *Sisyrinchium*, as well as to the genera *Calydorea* and *Herbertia*. These developed microsatellite markers can be used as an efficient tool for characterization of genetic variability in species belonging to Iridoideae, as well as for studies on population dynamics, genetic structure, and mating system in other *Sisyrinchium* species.

Key words: *Sisyrinchium palmifolium*; *Sisyrinchium marchioides*; Genetic diversity; Iridaceae; Microsatellite

INTRODUCTION

South America has been suggested as the center of origin and distribution of *Sisyrinchium* L. (Iridaceae: Iridoideae: Sisyrinchieae) species (Chauveau et al., 2011). *Sisyrinchium* species occur in naturally fragmented habitats, which can lead to low gene flow and isolation among populations. Within the past century, species of this genus have undergone additional fragmentation due to human activities, which has resulted in isolated subpopulations. The study of the genus *Sisyrinchium* is highly complex owing to the lack of reliable morphological apomorphies (Henderson, 1976), unresolved phylogenetic relationships at the species level (Chauveau et al., 2011; Karst and Wilson, 2012), reticulate evolution driven by polyploidy (Tacuatiá et al., 2012a,b; Alves et al., 2014), and hybridization (Henderson, 1976; Cholewa and Henderson, 1984; Yamaguchi and Hirai, 1987). The most complete phylogenetic study of *Sisyrinchium* performed by Chauveau et al. (2011) strongly suggests that species classified in section *Hydastylus* and section *Viperella* belong to a single group of plants that has undergone a recent adaptive radiation (Clade IV). In addition, the putative occurrence of incomplete lineage sorting within this clade may be partly explained by the low level of discrimination observed between species. Furthermore, Alves et al. (2014) also had difficulties assigning species identifications in this taxonomic group using plant barcode markers (ITS, *mat*K, and *trn*H-*psb*A). The authors suggest natural hybridization as one possible explanation because it can reduce the efficiency of DNA barcoding since it generally fails to identify species undergoing extensive reticulation. *Sisyrinchium palmifolium* L. and *S. marchioides* Spreng have great morphological similarity in flowers traits which may be attributable to a recent adaptive radiation of *Sisyrinchium* species (Alves et al., 2014). In earlier taxonomical studies, *S. palmifolium* was classified in the section *Hydastylus* and *S. marchioides* in the section *Viperella* (Ravenna, 2002). Both species are appreciated as ornamental plants, and therefore, wild populations are invariably subjected to habitat loss and

fragmentation into subpopulations (Ravenna, 2002; Overbeck et al., 2007). Knowledge on the patterns of genetic diversity and gene flow among species in this clade is essential to elucidate the genetic structure and mating system of these species and to provide genetic indicators supporting possible conservation strategies.

Therefore, the main goal of this study was to develop a set of polymorphic microsatellite (simple sequence repeats; SSR) markers compatible for both species (*S. palmifolium and S. marchioides*) to ensure that variability was revealed by the SSR markers. Further, cross-amplification was tested between these species and other species of Clade IV. In this study, an efficient molecular tool to investigate genetic variability of these closely related species is presented.

MATERIAL AND METHODS

Plant collection and DNA extraction

A total of 22 individuals from 3 different subpopulations of *S. marchioides* (ESC580, ESC562, and LBC5) and 20 individuals from 4 subpopulations of *S. palmifolium* (ESC193, ESC469, ESC586, and AITA19) were used to evaluate SSR polymorphism (**Table S1**). All primers were tested for these species on between 5 and 10 individuals from each subpopulation to examine their genetic variability. Voucher specimens were deposited in the ICN Herbarium, Instituto de Biociências, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil. Additional information on the populations and vouchers are provided in **Table S1**. Genomic DNA was isolated from dried leaves using a modified cetyltrimethylammonium bromide (CTAB) protocol (Doyle and Doyle, 1987).

Microsatellite-enriched library construction and sequencing

Enriched microsatellite libraries were constructed for *S. palmifolium* (ESC132) and *S. marchioides* (ESC123) according to the protocol described by Billote et al. (1999). Genomic DNA was digested using *Rsa*I (Invitrogen, Carlsbad, CA, USA), and subsequently enriched with biotinylated probes, $(CT)_8$ and $(GT)_8$. The fragments obtained were amplified by PCR, and amplification products were cloned into a pGEM T-Easy vector (Promega Corporation, Madison, WI, USA) before being transformed in competent XL1-blue *Escherichia coli* cells (Promega Corporation). A total of 96 recombinant colonies were obtained. For each library, 48 clones were sequenced bi-directionally in an automated ABI PRISM 377 sequencer (Perkin Elmer, Applied Biosystems, Foster City, CA, USA) using T7 and SP6 primers and BigDye terminator version 3.1 (Perkin Elmer-Applied Biosystems). From 48 sequenced clones of *S. marchioides* (ESC 123), 46 resulted in high quality sequences, 25 of which contained microsatellites. From these sequences, 12 primer sets were selected for validation in *S. marchioides*. For *S. palmifolium* (ESC 132), only 23 good sequences were obtained from 48 sequenced clones. Microsatellites were found in nine of these clones, from which four sets of primers were selected to be tested in *S. palmifolium.* Sequences were aligned and edited using the Seqman software (DNAStar, Madison, WI, USA). Repetitive regions were searched according to the methods outlined by Temnykh et al. (2001). From 16 clones (12 clones of *S. marchioides* and 4 of *S. palmifolium*) containing microsatellite repeats suitable for primer design, 32 primers (24 primers of *S. marchioides* and 8 of *S. palmifolium*) were designed using the Primer3 software (Rozen and Skaletsky, 1999). For each SSR, the forward primer was

synthesized with a 19 base-pair M13 tail (5'-CACGACGTTGTAAAACGAC-3') following Schuelke (2000)'s method, which involved three primers: a forward SSR-specific primer with the M13 tail at its 5' end, a reverse locus-specific primer, and a universal M13 primer labeled with the fluorescent dye 6-FAM (Perkin Elmer-Applied Biosystems).

PCR was performed using an Applied Biosystems Veriti thermocycler in 10-µL reaction volumes containing: 30 ng DNA template, 1X Taq DNA buffer, 2 mM $MgCl_2$, 100 µm dNTPs, 5 pmol forward primer, 10 pmol reverse primer, 1 pmol universal M13 primer, and 1U Taq polymerase (CenBiot, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil). A "touchdown" cycling program was used: 95°C for 3 min, followed by 10 cycles of 94°C for 30 s, 58°C decreasing to 48°C at 1°C per cycle for 30 s, 72°C for 30 s, 30 cycles of 94°C for 30 s, 48°C for 30 s, 72°C for 30 s, followed by a final extension of 10 min at 72°C. Loci were genotyped on an ABI 3730 DNA Analyzer Sequencer and sized against a 500 LIZ molecular size standard using the GeneMarker software (SoftGenetics, State College, PA, USA). Observed (H_O) and expected (H_E) heterozygosities, Shannon-Weiner diversity index (H'), and Nei's measure of population differentiation (G_{ST}) were calculated using GENEPOP version 4.2 (Raymond and Rousset, 1995) and MSA (Dieringer and Schlötterer, 2003) for *S. palmifolium*. However, the ATETRA program (Van Puyvelde et al., 2010) was used for the analysis of *S. marchioides* because this species showed a genotypic profile of two to four alleles per locus, indicating a polyploid status for this species, but its status was not confirmed through determination of the chromosome number.

Transferability tests were performed using the aforementioned PCR conditions. Amplification products were visualized on 1.5% agarose gels, and the presence or absence of bands was qualitatively scored.

RESULTS AND DISCUSSION

Microsatellite sequences-polymorphism

A total of 16 loci (12 from *S. marchioides* and four from *S. palmifolium*) were isolated, although only ten were polymorphic. Of the ten polymorphic loci, seven were from *S. marchioides* and three were from *S. palmifolium* (Table 1). Tests of linkage disequilibrium and Hardy-Weinberg equilibrium were not performed because each accession was considered as a subpopulation since the limits of the populations were not clear. It is important to mention that the actual distribution of the species was very modified by the fragmentation process, so it is possible that we analyzed only a portion of each population.

All seven loci from *S. marchioides* exhibited a maximum of four alleles per individual, suggesting tetraploidy, while the three loci observed for *S. palmifolium* exhibited no more than two alleles per individual. In *S. marchioides*, 59 alleles (Table 1) were identified with a minimum and maximum of two (VD3) and 15 (VB6) alleles per locus. In *S. palmifolium*, 28 alleles were identified, ranging from seven (PA5) to 11 (PD3) alleles per locus.

Values of the H' ranged from 0.678 to 2.891 with an average of 1.588 for *S. marchioides* and from 1.176 to 2.139 with a mean-value of 1.913 for *S. palmifolium*. In *S. marchioides*, the H_O and H_E ranged from 0.00 to 0.86 and from 0.00 to 0.90, respectively. In *S. palmifolium*, values ranged from 0.00 to 1.00 and from 0.75 to 1.00, respectively (Table 2). The G_{ST} values for *S. marchioides* and *S. palmifolium* were 0.033 and 0.295, respectively, indicating a higher differentiation among subpopulations of *S. palmifolium* than among subpopulations of *S.*

marchioides. A total of 9 loci were considered moderately to highly informative and therefore, adequate for genetic diversity studies. However, the VD3 locus did not exhibit differentiation among subpopulations.

Table 1. Characteristics of 10 microsatellite marker loci isolated from *Sisyrinchium marchioides* (V) and *S. palmifolium* (P), and GenBank accession No. (DNA sequences).

Locus name	Primer sequence (5'-3')	Repeat motif	No. of alleles	Size range (bp)	GenBank accession No.
VA5	F: CTTTGTCATCAGAGCTTCATGTGC* R: GTCATCTCGTTGCAGCCACCT	(TA)6(GT)7	9	173-191	KT456274
VA8	F: CCCAGGGGGAATTCAGAGTTAT* R: GGCTCCTATTGTCAGCTTGATG	(GT)22(GA)5(GT)4	12	206-232	KT456275
VB5	F: GTCCGCAAAAAGGTGAGCAAAT* R: CGGAACAATCGAACAAGTGACA	(CA)9	5	215-246	KT456276
VB6	F: CGATTGCCGATACGCCATAAA* R: ATGTTGTCTTCCCCCTCCATCA	(GA)13	15	190-246	KT456277
VC6	F: TGCTGTCAGTTGGGAATCATTG* R: GGCAGCAGCATCAACAGCAT	(GCT)5	12	186-225	KT456278
VD2	F: CAGTGAGGTCAGTGTTGCTT* R: GTCTTGGTTGTGTTTTGTTG	(TTA)6	4	145-162	KT456279
VD3	F: CGATACAAATAAAT* R: TGTATATATGGACGTTGTGGAC	(CA)5	2	172-194	KT456280
PA5	F: AAGCTCACAGCATACTTGATAAGG* R: TGTGAAGGAAGATGGATCTGAA	(GT)7	7	184-197	KT456281
PD3	F: CCACTTACTACCCCGAACTGTA* R: GGAGGAGTTGAGAAGACTTGTG	(CA)7	11	179-213	KT456282
PD6	F: CTGATTCGCAAGTGCATGA* R: CCCGGGATACAAAAACCTA	(CA)4CG(CA)16(TA)6	10	161-199	KT456283

*Primers that were M13-tailed at the 5' end.

Table 2. Genetic properties of the ten newly developed microsatellite markers for *Sisyrinchium*.

Sisyrinchium marchioides										*Sisyrinchium palmifolium*												
Locus	São Francisco de Paula (N = 10)			Caxias do Sul (N = 6)			Pelotas (N = 6)			Locus	Viamão (N = 5)			Aceguá (N = 5)			Porto Alegre - Morro Santana (N = 5)			Porto Alegre - Morro do Osso (N = 5)		
	A	H_O	H_E	A	H_O	H_E	A	H_O	H_E		A	H_O	H_E	A	H_O	H_E	A	H_O	H_E	A	H_O	H_E
VA5	7	0.75	0.77	5	0.77	0.81	5	0.80	0.84	PA5	4	0.80	0.75	4	1.00	0.82	6	1.00	0.87	5	1.00	0.86
VA8	6	0.80	0.82	9	0.86	0.89	9	0.86	0.90	PD3	6	0.80	0.89	5	1.00	0.80	5	0.80	0.86	5	0.80	0.88
VB5	5	0.18	0.19	3	0.00	0.00	3	0.00	0.00	PD6	4	0.00	1.00	4	0.60	0.78	4	0.20	0.80	4	0.60	0.75
VB6	4	0.53	0.55	9	0.77	0.81	8	0.80	0.83													
VC6	8	0.79	0.81	7	0.81	0.85	7	0.76	0.79													
VD2	2	0.47	0.48	3	0.51	0.53	3	0.49	0.51													
VD3	2	0.48	0.50	2	0.47	0.52	2	0.41	0.55													

N = number of individuals sampled; A = number of alleles sampled; H_O = observed heterozygosity; H_E = expected heterozygosity.

Transferability of SSR markers

Cross-species amplification of ten primers was tested in other *Sisyrinchium* species and also in species from two other genera. Sampling encompassed a total of 22 accessions, including 16 *Sisyrinchium* species (including *S. palmifolium* and *S. marchioides*), three *Calydorea* Herb. species (Iridaceae: Iridoideae: Tigridieae), and three *Herbertia* Sweet species (Iridaceae: Iridoideae: Tigridieae) (Table 3). The markers exhibited positive cross-amplification, with cross-amplification percentage values ranging from 28% (PD3) to 96% (VD2) with an average of 77%. The most transferability was obtained using primers isolated from *S. marchioides*. All primers exhibited higher rates of cross-amplification for species belonging to Clade IV of *Sisyrinchium*, including species of the section *Hydastylus* (Aita19, ESC193, ESC200, ESC240, ESC284, ESC382, ESC464, and ESC469), as well as of the section

Viperella (ESC157, ESC239, ESC248, ESC252, ESC263, and ESC318); the values varied from 50% (PD3) to 100% (VA5, VD2, and VD3), with an average of 82%. One individual of each subpopulation (ESC 193, ESC 469, ESC 586, and Aita 19) of *S. palmifolium* were also genotyped for loci that showed successful amplification (VA5, VA8, VB6, VC6, VD2, and VD3). The number of alleles found were 7, 6, 2, 2, 4, 3, and 2, respectively, indicating polymorphic loci. However, in this study, a total of 9 SSR primers had successful amplification and were polymorphic for *S. palmifolium.*

Table 3. Cross-amplification of ten microsatellite markers isolated from *Sisyrinchium marchioides* and *S. palmifolium* across 28 accessions of Iridaceae species.

Species	Sample	VA5	VA8	VB5	VB6	VC6	VD2	VD3	PA5	PD3	PD6
Calydorea campestris (Klatt) Baker	ESC632	+	W	+	+	+	+	+	-	-	W
Calydorea crocoides Ravenna	ESC684	+	-	+	-	+	+	+	-	-	+
Calydorea crocoides Ravenna	ESC688	+	W	+	+	+	+	+	-	-	+
Herbertia quareimana Ravenna	ESC520	-	-	-	-	-	-	-	-	-	-
Herbertia lahue ssp *lahue* (Molina) Goldblatt	ESC488	+	W	+	+	+	+	+	-	-	+
Herbertia sp	ESC521	+	W	+	+	+	+	+	-	-	W
Sisyrinchium aff. *luzula* Klotzsch ex Klatt	ESC678	+	+	+	+	+	+	+	+	-	+
Sisyrinchium aff. *marchioides* Ravenna	ESC157	+	+	+	W	+	+	+	W	-	+
Sisyrinchium. alatum Hook.	ESC239	+	+	+	+	+	+	+	+	+	W
Sisyrinchium alatum Hook.	ESC318	+	-	+	-	W	+	W	-	-	W
Sisyrinchium. balansae Baker	ESC464	+	+	+	+	+	+	+	W	-	+
Sisyrinchium cf. *balansae* Baker	ESC560	+	+	+	+	+	+	+	+	+	-
Sisyrinchium. cf. *scariosum* Klotzsch ex Klatt	ESC689	+	+	+	+	+	+	+	+	-	+
Sisyrinchium commutatum Klatt	ESC331	+	+	+	W	+	+	+	W	-	+
Sisyrinchium decumbens Ravenna	ESC200	+	+	+	W	+	+	+	+	-	+
Sisyrinchium fiebrigii I.M.Johnst.	ESC352	W	-	+	-	W	+	+	-	-	W
Sisyrinchium nidulare (Hand.-Mazz.) I.M. Johnst.	ESC240	W	-	W	W	-	+	+	-	-	-
Sisyrinchium palmifolium L.	Aita19	+	+	-	-	-	+	+	+	+	+
Sisyrinchium palmifolium L.	ESC 469	+	+	-	+	-	+	+	+	+	+
Sisyrinchium palmifolium L.	ESC193	+	+	-	-	+	+	+	+	+	-
Sisyrinchium palmifolium L.	ESC586	+	+	-	+	+	+	+	+	+	+
Sisyrinchium palmifolium L. subsp *giganteum* Ravenna.Foster	ESC382	W	+	+	W	+	+	+	-	+	W
Sisyrinchium rectilineum Ravenna	ESC284	+	+	+	+	+	+	+	+	+	+
Sisyrinchium restioides Spreng.	ESC252	+	W	+	+	+	+	+	W	-	+
Sisyrinchium setaceum Klatt	ESC690	+	+	+	W	+	+	+	+	-	+
Sisyrinchium vaginatum Spreng. ssp *vaginatum*	ESC263	+	+	+	+	+	+	+	W	-	+
Sisyrinchium weirii Baker	ESC248	+	+	+	-	+	+	+	+	-	-
Sisyrinchium sp (group Luzula new species)	ESC278	-	-	-	-	-	W	-	-	-	-
Transferability of SSR marker loci (%)	Species	92.0	78.0	78.0	71.0	82.0	96.0	92.0	60.0	28.0	92.0

+ = successful amplification; W = weak amplification; - = unsuccessful amplification.

CONCLUSIONS

To the best of our knowledge, this is the first study to report microsatellite markers for *S. palmifolium* and *S. marchioides*. The results indicate the effectiveness of the developed microsatellite loci for the characterization of genetic diversity in both species, which may help to discriminate the species of Clade IV of *Sisyrinchium*. These markers represent an excellent tool for future investigations of genetic diversity, taxonomy, systematics, and phylogeny of *Sisyrinchium* species, and other species belonging to Iridaceae. The primers developed for *S. marchioides* were quite efficient in transferability within Iridoideae. This result is very

important since these primers can be used in this group of plants in a comprehensive manner enabling genetic studies of populations, since most species are native and inhabit regions with fragmentation issues.

Conflicts of interest

The authors declare no conflict of interest.

ACKNOWLEDGMENTS

The authors would like to thank C. Zanella and M. Goetze for analytical support. Research supported by Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) and Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP).

REFERENCES

Alves TLS, Chauveau O, Eggers L and de Souza-Chies TT (2014). Species discrimination in *Sisyrinchium* (Iridaceae): assessment of DNA barcodes in a taxonomically challenging genus. *Mol. Ecol. Resour.* 14: 324-335. http://dx.doi.org/10.1111/1755-0998.12182

Billote N, Lagoda PJR, Risterucci AM and Baurens FC (1999). Microsatellite-enriched libraries: applied methodology for the development of SSR markers in tropical crops. *Fruits* 54: 277-288.

Chauveau O, Eggers L, Raquin C, Silvério A, et al. (2011). Evolution of oil-producing trichomes in *Sisyrinchium* (Iridaceae): insights from the first comprehensive phylogenetic analysis of the genus. *Ann. Bot. (Lond.)* 107: 1287-1312. http://dx.doi.org/10.1093/aob/mcr080

Cholewa AF and Henderson DM (1984). Biosystematics of *Sisyrinchium* section *Bermudiana* (Iridaceae) of the Rocky Mountains. *Brittonia* 36: 342-363. http://dx.doi.org/10.2307/2806596

Dieringer D and Schlötterer C (2003). Microsatellite analyzer (msa): a platform independent analysis tool for large microsatellite data sets. *Mol. Ecol. Notes* 3: 167-169. http://dx.doi.org/10.1046/j.1471-8286.2003.00351.x

Doyle JJ and Doyle JL (1987). A rapid DNA isolation procedure for small quantities of fresh leaf tissue. *Phytochem. Bull.* 19: 11-15.

Henderson DM (1976). A biosystematic study of Pacific Northwestern blue-eyed grasses (*Sisyrinchium*, Iridaceae). *Brittonia* 28: 149-176. http://dx.doi.org/10.2307/2805828

Karst L and Wilson CA (2012). Phylogeny of the New World genus *Sisyrinchium* (Iridaceae) based on analyses of plastid and nuclear DNA sequence data. *Syst. Bot.* 37: 87-95. http://dx.doi.org/10.1600/036364412X616666

Overbeck GE, Müller SC, Fidelis A, Pfadenhauer J, et al. (2007). Brazil's neglected biome: the South Brazilian Campos. *Perspect. Plant Ecol. Evol. Syst.* 9: 101-116. http://dx.doi.org/10.1016/j.ppees.2007.07.005

Ravenna P (2002). Revisional studies in the genus *Sisyrinchium* - VIII. *Onira* 6: 48.

Raymond M and Rousset F (1995). GENEPOP (version 1.2): population genetics software for exact tests and ecumenicism. *J. Hered.* 82: 648-649.

Rozen S and Skaletsky HJ (1999). Primer3 on the WWW for general users and for biologist programmers. In: Methods in molecular biology, vol. 132: bioinformatics methods and protocols (Misener S and Krawetz SA, eds.). Humana Press, Totowa.

Schuelke M (2000). An economic method for the fluorescent labeling of PCR fragments. *Nat. Biotechnol.* 18: 233-234. http://dx.doi.org/10.1038/72708

Tacuatiá LO, Souza-Chies TT, Flores AM, Eggers L, et al. (2012a). Cytogenetic and molecular characterization of morphologically variable *Sisyrinchium micranthum* (Iridaceae) in southern Brazil. *Bot. J. Linn. Soc.* 169: 350-364. http://dx.doi.org/10.1111/j.1095-8339.2012.01229.x

Tacuatiá LO, Cidade FW, Souza AP and Souza-Chies TT (2012b). Development and characterization of nine microsatellite loci for *Sisyrinchium micranthum* (Iridaceae). *Am. J. Bot.* 99: e402-e404. http://dx.doi.org/10.3732/ajb.1200105

Temnykh S, DeClerck G, Lukashova A, Lipovich L, et al. (2001). Computational and experimental analysis of microsatellites in rice (Oryza sativa L.): frequency, length variation, transposon associations, and genetic marker potential. *Genome Res.* 11: 1441-1452. http://dx.doi.org/10.1101/gr.184001

Van Puyvelde K, VAN Geert A and Triest L (2010). Atetra, a new software program to analyse tetraploid microsatellite data: comparison with tetra and tetrasat. *Mol. Ecol. Resour.* 10: 331-334. http://dx.doi.org/10.1111/j.1755-0998.2009.02748.x

Yamaguchi H and Hirai S (1987). Natural hybridization and flower color inheritance in *Sisyrinchium rosulatum Bickn. Weed Res.* 32: 38-45.

Supplementary material

Table S1. Accessions of *Sisyrinchium* collected from southern Brazil.