Novel Microsatellites for Calibrachoa heterophylla (Solanaceae) Endemic to the South Atlantic Coastal Plain of South America

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Primer Note

Novel microsatellites for *Calibrachoa heterophylla* (Solanaceae) endemic to the South Atlantic Coastal Plain of South America[1]

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- *Premise of the study: Calibrachoa heterophylla* (Solanaceae) is a petunia species restricted to the South Atlantic Coastal Plain of South America and presents a recent history of colonization from continental to coastal environments and diversification following the formation of the Coastal Plain during the Quaternary period.
- *Methods and Results:* This study reports a suite of 16 microsatellite loci for *C. heterophylla*. The applicability of these markers was assessed by genotyping 57 individuals from two natural populations. Of the 16 described loci, 12 were found to be polymorphic. Successful cross-amplification tests were obtained using 12 *Calibrachoa* species.
- *Conclusions:* The development of microsatellite markers will be useful to recover the contemporary history of the colonization of the Coastal Plain and to provide information for the conservation of this endemic species.

Key words: *Calibrachoa heterophylla*; cross-amplification; population genetics; simple sequence repeat (SSR) markers; Solanaceae; wild petunia.

Calibrachoa heterophylla (Sendtn.) Wijsman (Solanaceae) is a wild petunia species restricted to the South Atlantic Coastal Plain (SACP) of South America (Greppi et al., 2013; Mäder et al., 2013). The species is a melittophilous shrub that inhabits dunes or sandy grasslands of lakeside or seaside environments and possesses a conspicuous phenotypic plasticity that has been related to environmental differences in geographic distribution (Mäder et al., 2013). *Calibrachoa heterophylla* has a chromosome count of $2n = 18$ (Mishiba et al., 2000). Previous phylogeographic analyses showed that *C. heterophylla* has a continental origin and the deposition of coastal plains during the Quaternary period allowed the species to colonize the SACP (Mäder et al., 2013).

New challenges have emerged, such as the need to assess the contemporary patterns of genetic structure due to the secondary contact of basal genetic lineages after colonization of the SACP; to identify differences in the interpopulation gene flow patterns related to geographical and ecological barriers along the SACP; and to propose conservation measures considering that *C. heterophylla* could undergo drastic habitat reduction due to human-induced global climatic changes. New microsatellite (simple sequence repeat [SSR]) markers will be helpful in further studies that address these questions.

This is the first time that SSR markers have been developed for the genus *Calibrachoa* Cerv. Bossolini et al. (2011) and Kriedt et al. (2011) described several primer sets for the closely related genus *Petunia* Juss., which have been useful for answering evolutionary questions (e.g., Segatto et al., 2014).

METHODS AND RESULTS

Genomic DNA was extracted from the silica-dried leaves from one individual of *C. heterophylla* (geographic coordinates: 30°25′13″S, 51°13′30″W; herbarium voucher BHCB 116994) using a cetyltrimethylammonium bromide (CTAB) protocol according to Roy et al. (1992). An enriched library methodology was used to isolate specific repeat motifs according to Beheregaray et al. (2004). For this, genomic DNA was digested with the restriction enzyme *Rsa*I, and the fragments were linked to two oligo-adapters and amplified by PCR using a thermocycler (Applied Biosystems, Foster City, California, USA). The PCR conditions were as follows: initial denaturation at 95°C for 4 min, followed by 20 cycles of 94°C for 30 s, 60°C for 1 min, and 72°C for 1 min, and a final extension cycle at 72°C for 8 min. The products were purified using the QIAquick PCR Purification Kit (QIAGEN, Hilden, Germany), enriched for three motifs [$(dAT)_8$, $(dGA)_8$, and $(dGAA)_8$], and selectively captured using streptavidin magnetic particles (Invitrogen, Carlsbad, California, USA). PCR was performed on the microsatellite-enriched eluate using one of the oligo-adapters as a primer, with an initial denaturation at 95°C for 1 min, followed by 25 cycles of 94°C for 40 s, 60°C for 1 min, and 72°C for 2 min, and a final extension cycle at 72°C for 5 min. The enriched library was purified, cloned into the pGEM-T vector (Promega Corporation, Madison, Wisconsin, USA), and transformed into competent XLl-Blue *E. coli*. A total of 188 positive clones were PCR-amplified using M13(–20) forward and M13(–40) reverse primers, with an initial denaturation at 95°C for 4 min, followed by 30 cycles of 94°C for 30 s, 52°C for 45 s, and 72°C for 1 min, and a

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TABLE 1. Characteristics of the 16 microsatellite loci developed for *Calibrachoa heterophylla*.[a]

Locus	Primer sequences (5′–3′)	Repeat motif	Allele size range (bp)	T_a (°C)	Fluorescent dye[b]	GenBank accession no.
Che18	F: TTAAGGGAGGTGTAGCCCCA	$(CTT)_{21}$	149–179	54	HEX	KP091702
	R: ACAAAGAGATACATATACGCATGTGT					
Che46	F: TCAAGATAGCACCTTGTTTGCA	$(GA)_{28}$	223–249	54	HEX	KP091703
	R: CGTGCATACATGGTTAATTGGCT					
Che59	F: TCCTCTTCTTCGCTTGCTCC	$(CT)_7$	110–120	54	FAM	KP091704
	R: ATAAATTGGGACGGACGGGG					
Che119	F: TCCAACTGCAGTCAAGCTCT	$(CT)_{12}(TC)_{15}$	162–186	54	HEX	KP091705
	R: ACTGATGACCAATAGAGGAAGAACC					
Che26	F: ACGAAGCTTGTTACCAATCTCAAAA	$(AAG)_7$	90–102	54	HEX	KP091706
	R: AGCCAAAGTAGGGACGTTGA					
Che34	F: TCTTGAAGCCAATTGGAGAATAGT	$(TC)_{10}$	216–226	50	NED	KP091707
	R: TCGATCTGTGCTGCACATCA					
Che81	F: GACTACAGATTGGTCAACTTTTGAG	$(CT)_{16}$	322–358	56	FAM	KP091708
	R: AGGAGAGGCTTCTTTGGACA					
Che82	F: AGAAAAGAGGGATGAGGAGAACT	$(GA)_8$	127–131	49	NED	KP091709
	R: CTCGTCATTTTTCCTTGTCCCA					
Che85	F: TGGTAATGGAGCACGAGGAAG	$(TG)_6(GA)_8$	297–305	49	FAM	KP091710
	R: GGCTTTCAACTTTGTTCAAAACCC					
Che72	F: GCTGAGAACCAAGGAACAGC	$(GA)_{18}$	152–164	48	FAM	KP091711
	R: TCGATCTCTCATCCCCTGCA					
Che126	F: AGAGTTGACCCAAATTTCCCT	$(GA)_{16}$	338–366	48	NED	KP091712
	R: TCCTGTCTTGCCTTGTTTTCAC					
Che33	F: CCAAAGGATGAGGCATGCATTT	$(GA)_{20}$	184–210	50	FAM	KP091713
	R: CCAAACAATGCAGATCCCAAGT					
Che12	F: AACCCCTTCCCTCCAAACAC	$(TCT)_6$	291	54	NED	KP091714
	R: ACCTGTTATGGATTTCAAATGGAGT					
Che48	F: AGCAAGCTTGTCAGACGAAGA	$(GAA)_6$	139	54	NED	KP091715
	R: TTTCATGCTGGTCCATCCCC					
Che83	F: TTGAAGAGGAGGAGGAGGAG	$(AGA)_9$	175	55	FAM	KP091716
	R: AATGAATTCCAAGATCCAAGC					
Che114	F: TCATCAGTGGGAGGTTCATCAC	$(CTT)_{23}$	277	54	HEX	KP091717
	R: ACCATCTGAAAGTGAAAGCGAAG					

Note: T_a = annealing temperature.

[a]All values are based on 57 samples from Brazilian populations representing northern and southern regions of the South Atlantic Coastal Plain ($N = 22$ and $N = 35$, respectively).

[b]Fluorescent dye used for fragment analysis.

final extension cycle at 72°C for 8 min. The PCR products were purified and sequenced with a MegaBACE 1000 automated sequencer (GE Healthcare Biosciences, Pittsburgh, Pennsylvania, USA). Forty clones possessed SSRs, of which 27 were adequate for primer design using Primer3 (Untergasser et al., 2012), with primer sizes between 18 and 25 bp, GC contents ranging from 48% to 60%, and melting temperatures varying from 55°C to 65°C.

TABLE 2. Genetic properties of the 16 microsatellite loci developed for *Calibrachoa heterophylla*.

	Santo Antônio da Patrulha[a] ($n = 22$)			Santa Vitória do Palmar[a] ($n = 35$)		
Locus	A	H_o	H_e	A	H_o	H_e
Che18	5	0.1875	0.73185	4	0.47059	0.60843
Che46	9	0.7	0.83205	12	0.47059	0.88455
Che59	2	0.42105	0.34139	3	0.5	0.60623
Che119	7	0.77273	0.77484	4	0.62857	0.5735
Che26	4	0.40909	0.56977	3	0.02941	0.37357
Che34	3	0.54545	0.42706	1	0	0
Che81	3	0.3	0.44487	5	0.23529	0.63784
Che82	1	0	0	2	0.27586	0.50817
Che85	2	0.05263	0.14936	3	0.2963	0.36618
Che72	2	0.4	0.32821	5	0.66667	0.64149
Che126	5	0.10526	0.51351	9	0.3	0.79103
Che 33	5	0.2	0.71264	5	0.22222	0.66013
Che12	1	0	0	1	0	0
Che48	1	0	0	1	0	0
Che83	1	0	0	1	0	0
Che114	1	0	0	1	0	0

Note: A = number of alleles; H_e = expected heterozygosity; H_o = observed heterozygosity; n = number of individuals sampled.

[a]Santo Antônio da Patrulha population: Geographic coordinates = 29°53′34.5″S, 50°25′45.7″W; herbarium vouchers BHCB 104866/104867. Santa Vitória do Palmar population: Geographic coordinates = 32°59′15.5″S, 52°43′56.3″W; herbarium vouchers BHCB 104907/104908. Both populations are located in the South Atlantic Coastal Plain, Brazil.

TABLE 3. Cross-amplification results for the 16 microsatellite markers developed for *Calibrachoa heterophylla* in 95 individuals of 12 *Calibrachoa* species.

Species	ID LEM[a]	Che18	Che46	Che59	Che119	Che26	Che34	Che81	Che82	Che85	Che72	Che126	Che33	Che12	Che48	Che83	Che114
C. elegans (Miers) Stehmann & Semir	C.eleg 4	0	0	1	1	1	1	0	0	1	0	1	0	1	1	0	1
	C.eleg 10	0	1	1	1	1	1	0	0	1	0	1	0	1	1	0	1
	C.eleg 20	0	0	1	1	1	1	0	0	1	0	1	0	1	1	0	1
	C.eleg 30	0	0	1	1	1	1	0	0	1	0	1	0	1	1	0	1
	C.eleg 43	0	1	1	1	1	1	0	0	1	0	0	0	1	1	0	1
	C.eleg 50	0	1	1	1	1	1	0	0	1	0	1	0	1	1	0	1
	C.eleg 60	0	1	1	1	1	1	0	0	1	0	1	0	1	1	0	1
	C.eleg 70	0	1	1	1	1	1	0	0	1	0	0	0	1	1	0	1
C. ericifolia (R. E. Fr.) Wijsman	C.eric 1	0	0	1	1	0	1	0	0	0	0	1	0	1	1	0	1
	C.eric 15	0	0	1	1	1	1	0	0	0	0	1	0	1	1	0	1
	C.eric 65	0	1	1	1	1	1	0	0	0	0	1	0	1	1	0	1
	C.eric 92	0	0	1	1	1	1	0	0	0	0	1	0	1	1	0	1
	C.eric 107	0	0	1	1	1	1	0	0	0	0	1	0	1	1	0	1
	C.eric 148	0	0	1	1	1	1	0	0	0	0	1	0	1	1	0	1
	C.eric 179	0	1	1	1	1	1	0	0	0	0	1	0	1	1	0	1
	C.eric 180	0	1	1	1	1	1	0	0	0	0	1	0	1	1	0	1
C. excellens (R. E. Fr.) Wijsman subsp. *atropurpurea* Stehmann & Semir	C.exca 1	0	1	1	0	0	0	0	0	0	0	1	0	0	1	0	1
	C.exca 7	0	1	1	0	0	0	0	0	0	0	1	0	1	1	0	1
	C.exca 9	0	1	1	0	0	0	0	0	0	0	1	0	1	1	0	1
	C.exca 20	0	1	1	0	0	0	0	0	0	0	1	0	0	1	0	1
	C.exca 24	0	1	1	0	1	0	0	0	0	0	1	0	0	1	0	1
	C.exca 25	0	1	1	0	1	0	0	0	0	0	1	0	1	1	0	1
	C.exca 40	0	1	1	0	0	0	0	0	0	0	1	0	1	1	0	1
	C.exca 50	0	0	1	0	0	0	0	0	0	0	1	0	1	1	0	1
C. excellens (R. E. Fr.) Wijsman subsp. *excellens*	C.exce 2	0	1	0	1	0	1	0	0	0	0	1	0	0	1	0	0
	C.exce 9	0	0	0	1	0	1	1	0	0	0	1	0	0	1	0	0
	C.exce 12	0	0	1	1	1	1	0	0	0	0	1	0	0	1	0	1
	C.exce 30	0	0	1	1	1	1	1	0	0	0	1	0	1	1	0	1
	C.exce 40	0	0	1	1	1	1	1	0	0	0	1	0	1	1	0	1
	C.exce 100	0	0	1	1	1	1	1	0	0	0	1	0	1	1	0	1
	C.exce 120	0	0	1	1	1	1	1	0	0	0	1	0	1	1	0	1
	C.exce 220	0	0	1	1	1	1	1	0	0	0	1	0	1	1	0	1
C. humilis (R. E. Fr.) Stehmann & Semir	C.humi 3	0	0	1	1	1	1	1	0	0	0	1	0	0	1	0	1
	C.humi 4	0	0	1	1	1	1	1	0	0	0	1	0	1	1	0	1
	C.humi 15	0	0	1	1	1	1	1	0	0	0	1	0	1	1	0	1
	C.humi 21	0	0	1	1	0	1	1	0	0	0	1	0	0	1	0	1
	C.humi 25	0	0	1	1	0	1	1	0	0	0	1	0	0	1	0	1
	C.humi 30	0	0	1	1	1	1	0	0	0	0	1	0	0	1	0	1
	C.humi 35	0	0	1	1	1	1	1	0	0	0	1	0	0	1	0	1
	C.humi 37	0	0	1	1	1	1	0	0	0	0	1	0	0	1	0	1
	C.humi 42	0	0	1	1	1	1	0	0	0	0	1	0	0	1	0	1
	C.humi 44	0	0	1	1	1	1	1	0	0	0	1	0	0	1	0	1
	C.humi 47	0	0	1	1	0	1	1	0	0	0	1	0	0	1	0	1
	C.humi 55	0	0	1	1	0	1	1	0	0	0	1	0	0	1	0	1
	C.humi 64	0	0	1	1	0	1	1	0	0	0	1	0	0	1	0	1
C. linearis (Hook.) Wijsman	C.line 5	0	0	1	1	0	0	1	0	0	0	1	0	0	1	0	1
	C.line 7	0	0	1	1	0	0	1	0	0	0	1	0	0	1	0	1
	C.line 12	0	0	1	1	1	0	1	0	0	0	1	0	0	1	0	1

TABLE 3. Continued.

Species	ID LEM[a]	Che18	Che46	Che59	Che119	Che26	Che34	Che81	Che82	Che85	Che72	Che126	Che33	Che12	Che48	Che83	Che114
C. linoides (Sendtn.) Wijsman	C.lino 1	0	1	1	1	1	1	0	0	0	0	1	0	0	1	0	1
	C.lino 13	0	1	1	1	1	1	0	0	0	0	1	0	0	1	0	1
	C.lino 20	0	1	0	1	1	1	0	0	0	0	1	0	0	1	0	1
	C.lino 33	0	0	1	1	0	1	0	0	0	0	1	0	0	1	0	1
	C.lino 52	0	1	1	1	1	1	0	0	0	0	1	0	1	1	0	1
	C.lino 72	0	0	1	1	0	1	0	0	0	0	1	0	0	1	0	1
	C.lino 189	0	1	1	1	1	1	0	0	0	0	1	0	1	1	0	1
	C.lino 205	0	1	1	1	1	1	0	0	0	0	1	0	1	1	0	1
C. ovalifolia (Miers) Stehmann & Semir	C.oval 2	0	0	0	1	0	1	0	0	0	0	0	0	0	0	0	0
	C.oval 11	0	1	1	1	0	1	0	0	0	0	1	0	0	1	0	1
	C.oval 17	0	1	1	1	0	1	0	0	0	0	1	0	0	1	0	1
	C.oval 26	0	1	0	1	0	1	0	0	0	0	0	0	0	1	0	1
	C.oval 34	0	1	0	1	0	1	0	0	0	0	0	0	0	1	0	1
	C.oval 35	0	0	0	1	0	1	0	0	0	0	1	0	0	1	0	1
	C.oval 101	0	0	1	1	0	1	0	0	0	0	1	0	0	1	0	1
	C.oval 129	0	0	1	1	0	1	0	0	0	0	0	0	0	1	0	1
C. paranensis (Dusén) Wijsman	C.para 1	0	0	1	1	1	1	0	0	0	0	1	0	1	1	0	1
	C.para 28	0	0	1	1	1	1	0	0	0	0	1	0	0	1	0	1
	C.para 47	0	0	1	1	1	1	0	0	0	0	1	0	1	1	0	1
	C.para 73	0	0	1	1	1	1	0	0	0	0	1	0	1	1	0	1
	C.para 99	0	0	1	1	1	1	0	0	0	0	1	0	1	1	0	1
	C.para 125	0	0	1	1	1	1	0	0	0	0	1	0	1	1	0	1
	C.para 162	0	0	1	1	1	1	0	0	0	0	1	0	1	1	0	1
	C.para 186	0	0	1	1	1	1	0	0	0	0	1	0	0	1	0	1
C. pygmea (R. E. Fr.) Wijsman	C.pygm 1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	C.pygm 30	0	0	0	0	0	0	0	0	0	0	1	0	0	1	0	1
	C.pygm 31	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1
	C.pygm 36	0	0	0	0	0	0	0	0	0	0	1	0	0	1	0	1
	C.pygm 41	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	1
	C.pygm 51	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
	C.pygm 55	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
C. serrulata (L. B. Sm. & Downs) Stehmann & Semir	C.serr 1	0	0	1	1	0	1	0	0	1	0	1	0	1	1	0	1
	C.serr 3	0	0	1	1	0	1	0	0	1	0	1	0	1	1	0	1
	C.serr 5	0	0	1	1	0	1	0	0	1	0	1	0	1	1	0	1
	C.serr 7	0	0	1	1	0	1	0	0	1	0	0	0	1	1	0	1
	C.serr 11	0	0	1	1	0	1	0	0	1	0	1	0	1	1	0	1
	C.serr 15	0	0	1	1	0	1	0	0	1	0	1	0	1	1	0	1
	C.serr 17	0	0	1	1	0	1	0	0	1	0	1	0	0	1	0	1
	C.serr 19	0	0	1	1	0	1	0	0	1	0	1	0	1	1	0	1
C. thymifolia (A. St.-Hil.) Stehmann & Semir	C.thym 1	0	0	1	1	0	1	0	0	1	0	1	0	0	1	0	1
	C.thym 3	0	0	1	1	0	1	0	0	1	0	1	0	1	1	0	1
	C.thym 5	0	0	1	1	0	1	0	0	1	0	1	0	1	1	0	1
	C.thym 36	0	0	1	1	0	1	0	0	1	0	1	0	1	1	0	1
	C.thym 54	0	0	1	1	0	1	0	0	1	0	1	0	0	1	0	1
	C.thym 56	0	0	1	1	0	1	0	0	1	0	1	0	0	1	0	1
	C.thym 58	0	0	1	1	0	1	0	0	1	0	1	0	0	1	0	1
	C.thym 59	0	0	1	1	0	1	0	0	1	0	1	0	0	1	0	1

Note: 1 = successful amplification; 0 = failed amplification.

[a] Sample identification code in the Laboratory of Molecular Evolution, Department of Genetics, Universidade Federal do Rio Grande do Sul.

The resulting markers were tested in two populations of *C. heterophylla* belonging to two different chloroplast haplogroups (Mäder et al., 2013): Santo Antônio da Patrulha (geographic coordinates 29°53′34.5″S, 50°25′45.7″W; herbarium vouchers BHCB 104866/104867) and Santa Vitória do Palmar (geographic coordinates 32°59′15.5″S, 52°43′56.3″W; herbarium vouchers BHCB 104907/104908), Rio Grande do Sul, Brazil. PCR was performed in 10-μL reactions containing ~10 ng/μL of template DNA, 200 μM each dNTP (Invitrogen), 2 pmol fluorescently labeled M13(−21) primer and reverse primer, 0.4 pmol forward primer, 2.0 mM $MgCl_2$ (Invitrogen), 0.5 units of *Taq* Platinum DNA polymerase, and 1× *Taq* Platinum reaction buffer (Invitrogen). The PCR conditions were as follows: initial denaturation at 94°C for 3 min, followed by 32 cycles of 94°C for 20 s, 53–65°C for 45 s, and 72°C for 1 min, and a final extension cycle at 72°C for 10 min. The forward primers were labeled with FAM, NED, or HEX (Table 1). The products were analyzed using a MegaBACE 1000 automated sequencer with the ET-ROX 550 size ladder (GE Healthcare Biosciences). Genotyping results were scored using GeneMarker software (version 2.4; SoftGenetics, State College, Pennsylvania, USA).

Sixteen loci with a clear and strong single band for each allele were identified and used to genotype 57 individuals from two populations of *C. heterophylla*. Twelve loci displayed polymorphism, whereas the other four loci were monomorphic (Table 1). All of the individuals presented one or two alleles (consistent with the diploid condition of *C. heterophylla*) that matched the expected sizes based on cloned sequences. In the Santo Antônio da Patrulha population, the number of alleles per locus for the 12 polymorphic loci varied from one to nine, with an average of four, and observed (H_o) and expected (H_e) heterozygosity ranged from 0 to 0.773 and 0 to 0.832, with averages of 0.341 and 0.485, respectively (Table 2). In the Santa Vitória do Palmar population, the number of alleles per locus for the 12 polymorphic loci varied from one to 12, and H_o and H_e ranged from 0 to 0.667 and 0 to 0.885, with averages of 0.341 and 0.554, respectively (Table 2). Considering both populations, the total number of alleles per locus for the 12 polymorphic loci ranged from two (Che34 and Che82) to 13 (Che46), and H_o and H_e ranged from 0.138 to 0.701 and from 0.193 to 0.899, with averages of 0.341 and 0.624, respectively (Table 2). Che18, Che46, Che126, and Che33 deviated from HWE in the two populations, and Che26, Che81, Che82, and Che85 deviated from HWE in the Santa Vitória do Palmar population ($P < 0.04$), all due to heterozygote deficiency. All analyses were conducted with Arlequin version 3.5 (Excoffier and Lischer, 2010). There are no specific studies in reproductive biology for *C. heterophylla*, but we suspect that high levels of autogamy (observed in some *Petunia* species, e.g., Turchetto et al., 2015) could be responsible for the low levels of heterozygosity in the analyzed populations. Additionally, considering that *C. heterophylla* recently colonized and is in continuing expansion over the SACP, one would expect to find populations with relatively high allelic richness and, at the same time, low heterozygosity.

Cross-amplification of all the developed loci was tested in 95 individuals of 12 *Calibrachoa* species, covering most of the geographic range and phylogenetic diversity of the genus (Table 3, Appendix 1, Appendix S1; Fregonezi et al., 2012), under the same PCR conditions used for *C. heterophylla*. Except for *C. pygmea* (R. E. Fr.) Wijsman, most of the loci showed positive amplification in the species tested, indicating that the developed markers are useful for other *Calibrachoa* species. The markers Che59, Che119, Che34, Che126, Che48, and Che114 showed the highest rates of the cross-amplification tests (Table 3, Appendix S1). The lower rates of cross-amplification for *C. pygmea* are unsurprising given that this species is classified in a different subgenus and is phylogenetically more distant to *C. heterophylla* than the remaining species included in this study (Table 3, Appendix S1; Fregonezi et al., 2012).

CONCLUSIONS

These are the first SSR markers developed for *C. heterophylla*. These loci will allow us to investigate the effects of landscape heterogeneity on the genetic structure of *C. heterophylla* populations and, combined with other analyses and species, will allow us to understand the colonization process of plant groups to the SACP. These markers may also be valuable for conservation of this endemic species.

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Appendix 1. Locality and voucher information for *Calibrachoa* species used in this study.

Species	Locality[a]	ID LEM[b]	Herbarium voucher no.[c]	Latitude	Longitude
C. elegans (Miers) Stehmann & Semir	Serra da Calçada, Brumadinho/MG, Brazil	C.eleg 4	BHCB 45365	−20.09305556	−43.98361111
	Serra da Calçada, Brumadinho/MG, Brazil	C.eleg 10	BHCB 45365	−20.09305556	−43.98361111
	Santana do Garabéu/MG, Brazil	C.eleg 20	BHCB 103115	−21.61094722	−44.12620278
	Santana do Garabéu/MG, Brazil	C.eleg 30	BHCB 103115	−21.61094722	−44.12620278
	Morro do Chapéu, Nova Lima/MG, Brazil	C.eleg 43	NA	−20.10310278	−43.94620278
	Morro do Chapéu, Nova Lima/MG, Brazil	C.eleg 50	NA	−20.10310278	−43.94620278
	Retiro das Pedras, Brumadinho/MG, Brazil	C.eleg 60	BHCB 45365	−20.09305556	−43.98333333
	Retiro das Pedras, Brumadinho/MG, Brazil	C.eleg 70	BHCB 45365	−20.09305556	−43.98333333
C. ericifolia (R. E. Fr.) Wijsman	Passo do Pupo, Ponta Grossa/PR, Brazil	C.eric 1	BHCB 96546	−25.14690737	−49.95483021
	Ponta Grossa/PR, Brazil	C.eric 15	BHCB 104025	−25.25597504	−50.15140244
	Castro, Tibagi/PR, Brazil	C.eric 65	BHCB 104026	−24.76576497	−50.1578068
	Castro, Tibagi/PR, Brazil	C.eric 92	BHCB 104026	−24.76792733	−50.15422672
	Castro, Tibagi/PR, Brazil	C.eric 107	BHCB 104027	−24.65006797	−50.22478516
	Parque Estadual do Quartelá, Tibagi/PR, Brazil	C.eric 148	BHCB 104028	−24.56777939	−50.26055384
	Parque Estadual do Quartelá, Tibagi/PR, Brazil	C.eric 179	BHCB 104028	−24.56777939	−50.26055384
	Balsa Nova/PR, Brazil	C.eric 180	BHCB 104029	−25.44122239	−49.74743217
C. excellens (R. E. Fr.) Wijsman subsp. *atropurpurea* Stehmann & Semir	Pedra da Abelha, Pedra do Segredo, Caçapava do Sul/RS, Brazil	C.exca 1	BHCB 75119	−30.53129713	−53.55028538
	Bagé, Santana do Livramento/RS, Brazil	C.exca 7	BHCB 102126	−31.21294677	−54.28641303
	Pedra do Segredo, Caçapava do Sul/RS, Brazil	C.exca 9	NA	−30.5423423	−53.55549993
	Canguçu/RS, Brazil	C.exca 20	HUEFS 79276	−31.34311667	−52.67974444
	P.E. Itapuã, Viamão/RS, Brazil	C.exca 24	NA	NA	NA
	Loreto, São Vicente do Sul/RS, Brazil	C.exca 25	BHCB 102094	−29.72229746	−54.84055541
	Galpão de Pedra, Caçapava do Sul/RS, Brazil	C.exca 40	NA	−30.547039	−53.546476
	Galpão de Pedra, Pedra do Segredo, Caçapava do Sul/RS, Brazil	C.exca 50	ICN 158617	−30.989167	−53.645278
C. excellens (R. E. Fr.) Wijsman subsp. *excellens*	Pedra do Segredo, Caçapava do Sul/RS, Brazil	C.exce 2	BHCB 75090	−30.54944504	−53.54229307
	Pantano Grande, Encruzilhada do Sul/RS, Brazil	C.exce 9	BHCB 75150	−30.35481968	−52.40705791
	Bom Jardim da Serra/SC, Brazil	C.exce 12	BHCB 80084	−28.34020411	−49.62996039
	Estrada Urubici–São Joaquim, Urubici/SC, Brazil	C.exce 30	BHCB 96689	−28.14887971	−49.71661418
	Guarapuava/PR, Brazil	C.exce 40	BHCB 96605	−25.48049958	−51.53825009
	Pinhão/PR, Brazil	C.exce 100	BHCB 96612	−25.948983	−51.61110903
	Porto Alegre/RS, Brazil	C.exce 120	NA	−30.04383263	−51.11589396
	Xaxim/SC, Brazil	C.exce 220	BHCB 128573	−26.95230679	−52.51188593
C. humilis (R. E. Fr.) Stehmann & Semir	Uruguaiana/RS, Brazil	C.humi 3	BHCB 117015	−29.41827955	−56.69409838
	Uruguaiana/RS, Brazil	C.humi 4	BHCB 117015	−29.41827955	−56.69409838
	Quaraí/RS, Brazil	C.humi 15	Ana Luíza Cazé e Geraldo Mader	−30.31516667	−56.47583333
	Três Cerros, Uruguay	C.humi 21	Greppi 1470	−29.12433333	−56.896
	Três Cerros, Uruguay	C.humi 25	Greppi 1474	−29.12433333	−56.896
	Três Cerros, Uruguay	C.humi 30	Greppi 1479	−29.12433333	−56.896
	Três Cerros, Uruguay	C.humi 35	Greppi 1484	−29.12433333	−56.896
	Três Cerros, Uruguay	C.humi 37	Greppi 1486	−29.12433333	−56.896
	Três Cerros, Uruguay	C.humi 42	Greppi 1491	−29.12433333	−56.896
	Três Cerros, Uruguay	C.humi 44	Greppi 1493	−29.12433333	−56.896
	Mercedes-Corrientes, Argentina	C.humi 47	Greppi 1496	−29.40433333	−57.96833333
	Mercedes-Corrientes, Argentina	C.humi 55	Greppi 1510	−29.40433333	−57.96833333
	Monte Caseros, Argentina	C.humi 64	Greppi 1519	−30.250467	−57.638451
C. linearis (Hook.) Wijsman	Colon. Balneario San Jose, Argentina	C.line 5	Greppi 1023	−32.18333333	−58.16666667
	Corrientes, Argentina	C.line 7	NA	−29.21283333	−59.22025
	Corrientes, Argentina	C.line 12	NA	−29.57605556	−59.33047222

Appendix 1. Continued.

Species	Locality[a]	ID LEM[b]	Herbarium voucher no.[c]	Latitude	Longitude
C. linoides (Sendtn.) Wijsman	São Bento do Sul, Campo Alegre/SC, Brazil	C.lino 1	João Renato Stehmann 3313 (BHCB)	−26.20806629	−49.30657214
	Rio do Sul/SC, Brazil	C.lino 13	HUEFS 75248	−27.31232411	−50.38754739
	Otacílio Costa, Petrolândia/SC, Brazil	C.lino 20	João Renato Stehmann 3322 (BHCB)	−27.57700584	−50.00116462
	Paraíso da Serra/SC, Brazil	C.lino 33	João Renato Stehmann 3330 (BHCB)	−27.81610248	−49.54344749
	Bituruna/PR, Brazil	C.lino 52	ICN 161236	−26.16983668	−51.59984694
	General Carneiro, Matos Costa/SC, Brazil	C.lino 72	ICN 160338	−26.46892235	−51.17974578
	Monte Verde/MG, Brazil	C.lino 189	NA	−22.86653442	−46.04815029
	Monte Verde/MG, Brazil	C.lino 205	NA	−22.86827316	−46.04316213
C. ovalifolia (Miers) Stehmann & Semir	Pedra da Abelha, Pedra do Segredo, Caçapava do Sul/RS, Brazil	C.oval 2	BHCB 75116	−30.53078785	−53.54969655
	Morro Grande, Cachoeira do Sul/RS, Brazil	C.oval 11	BHCB 75136	−30.3199487	−52.92622084
	Santana do Livramento, Quaraí/RS, Brazil	C.oval 17	BHCB 79868	−30.80620235	−55.61801598
	Cerro do Jarau, Quaraí–Uruguaiana/RS, Brazil	C.oval 26	BHCB 79879	−30.19979135	−56.49454328
	Pedra do Segredo, Caçapava do Sul/RS, Brazil	C.oval 34	BHCB 75123	−30.5423423	−53.55549993
	Pedra do Segredo, Caçapava do Sul/RS, Brazil	C.oval 35	BHCB 75123	−30.5423423	−53.55549993
	S. Martinho da Serra, Júlio de Castilho/RS, Brazil	C.oval 101	BHCB 117004	−29.32271143	−53.67828031
	Morro da Cruz, Caçapava do Sul/RS, Brazil	C.oval 129	BHCB 75123	−30.896659	−53.421003
C. paranensis (Dusén) Wijsman	Serra de São Luis do Purunã, Campo Largo-Ponta Grossa/PR, Brazil	C.para 1	ICN 189546	−25.45953736	−49.64560739
	Serra de São Luis do Purunã, Balsa Nova/PR, Brazil	C.para 28	ICN 161233	−25.46811154	−49.65597966
	Serra de São Luis do Purunã, Balsa Nova/PR, Brazil	C.para 47	João Renato Stehmann 4211 (BHCB)	−25.46813099	−49.63903748
	Serra de São Luis do Purunã, Balsa Nova/PR, Brazil	C.para 73	João Renato Stehmann 4215 (BHCB)	−25.46581876	−49.72120914
	Passo do Pupo, Ponta Grossa/PR, Brazil	C.para 99	João Renato Stehmann 4222 (BHCB)	−25.14690737	−49.95483021
	Reserva Natural Buraco do Padre, Ponta Grossa/PR, Brazil	C.para 125	João Renato Stehmann 4227 (BHCB)	−25.17168009	−49.96811494
	Guarapuava, Laranjeiras do Sul/PR, Brazil	C.para 162	João Renato Stehmann 4259 (BHCB)	−25.41794392	−51.67142328
	Distrito de São Luiz do Purunã, Balsa Nova/PR, Brazil	C.para 186	BHCB 104021	−25.46323688	−49.71047686
C. pygmea (R. E. Fr.) Wijsman	Quaraí–Uruguaiana/RS, Brazil	C.pygm 1	BHCB 79881	−30.15518605	−56.43883799
	Alegrete/RS, Brazil	C.pygm 30	BHCB 102099	−29.7880923	−55.72627732
	Alegrete/RS, Brazil	C.pygm 31	BHCB 102099	−29.7880923	−55.72627732
	Alegrete/RS, Brazil	C.pygm 36	BHCB 102099	−29.7880923	−55.72627732
	Alegrete/RS, Brazil	C.pygm 41	BHCB 102099	−29.7880923	−55.72627732
	Alegrete/RS, Brazil	C.pygm 51	BHCB 102099	−29.7880923	−55.72627732
	Alegrete/RS, Brazil	C.pygm 55	BHCB 102099	−29.7880923	−55.72627732
C. serrulata (L. B. Sm. & Downs) Stehmann & Semir	Cânion da Serra do Rio do Rastro, Lauro Muller/SC, Brazil	C.serr 1	BHCB 99775	−28.38594625	−49.54480067
	Cânion da Serra do Rio do Rastro, Lauro Muller/SC, Brazil	C.serr 3	BHCB 02009	−28.3877839	−49.54396608
	Cânion da Serra do Rio do Rastro, Lauro Muller/SC, Brazil	C.serr 5	BHCB 02009	−28.3877839	−49.54396608
	Cânion da Serra do Rio do Rastro, Lauro Muller/SC, Brazil	C.serr 7	BHCB 02009	−28.3877839	−49.54396608
	Cânion da Serra do Rio do Rastro, Lauro Muller/SC, Brazil	C.serr 11	BHCB 02009	−28.3877839	−49.54396608
	Bom Jardim da Serra/SC, Brazil	C.serr 15	BHCB 116965	−28.36292275	−49.55145128
	Bom Jardim da Serra/SC, Brazil	C.serr 17	BHCB 116965	−28.36292275	−49.55145128
	Bom Jardim da Serra/SC, Brazil	C.serr 19	BHCB 116964	−28.35748858	−49.55059298
C. thymifolia (A. St.-Hil.) Stehmann & Semir	Federación Santa Ana, playa de Sta. Ana, Argentina	C.thym 1	Greppi 1003	−30.91305556	−57.91805556
	Entre Rios, Sta. Ana, playa arenosa sobre Rio Uruguay, Argentina	C.thym 3	Greppi 1017	−30.9	−57.91666667
	Santa Ana, Entre Rios, Argentina	C.thym 5	Greppi 1416	−30.899868	−57.931123
	Paso de Los Libres, Argentina	C.thym 36	Greppi 1447	−29.714594	−57.097374
	Colon, Entre Rios, Argentina	C.thym 54	Greppi 1502	−32.184	−58.17416667
	San Roque, Corrientes, Argentina	C.thym 56	Greppi 1504	−28.90638889	−58.66166667
	Concordia, Entre Ríos, Argentina	C.thym 58	Greppi 1506	−31.40183333	−58.10166667
	Concordia, Entre Ríos, Argentina	C.thym 59	Greppi 1507	−31.40183333	−58.10166667

[a] MG = Minas Gerais state; PR = Paraná state; RS = Rio Grande do Sul state; SC = Santa Catarina state.

[b] Sample identification code in the Laboratory of Molecular Evolution, Department of Genetics, Universidade Federal do Rio Grande do Sul.

[c] Herbarium acronyms are per Index Herbariorum (http://sweetgum.nybg.org/ih/); NA = not available. Vouchers identified as "Greppi" are greenhouse-cultivated samples provided by Julián Alejandro Greppi (Instituto Nacional de Tecnología Agropecuaria [INTA], Argentina).