New Microsatellite Markers for Wild and Commercial Species of Passiflora (Passifloraceae) and Cross-Amplification

Authors: Cerqueira-Silva, Carlos B. M., Santos, Elisa S. L., Vieira, João G. P., Mori, Gustavo M., Jesus, Onildo N., et al.

Source: Applications in Plant Sciences, 2(2)

Published By: Botanical Society of America

URL: https://doi.org/10.3732/apps.1300061

Applications in Plant Sciences 2014 2(2): 1300061

Primer Note

New microsatellite markers for wild and commercial species of *Passiflora* (Passifloraceae) and cross-amplification[1]

Carlos B. M. Cerqueira-Silva[2,3], Elisa S. L. Santos[2,3], João G. P. Vieira[3], Gustavo M. Mori[2], Onildo N. Jesus[4], Ronan X. Corrêa[5], and Anete P. Souza[2,6,7]

[2]Centro de Biologia Molecular e Engenharia Genética, Instituto de Biologia, Universidade Estadual de Campinas, CP 6010, 13083-875 Campinas, São Paulo, Brazil; [3]Departamento de Ciências Exatas e Naturais, Universidade Estadual do Sudoeste da Bahia, 45700-000 Itapetinga, Bahia, Brazil; [4]Empresa Brasileira de Pesquisa Agropecuária, Centro Nacional de Pesquisa em Mandioca e Fruticultura Tropical, 44380-000 Cruz das Almas, Bahia, Brazil; [5]Departamento de Ciências Biológicas, Universidade Estadual de Santa Cruz, Rodovia Ilhéus-Itabuna Km 16, 45662-900 Ilhéus, Bahia, Brazil; and [6]Departamento de Biologia Vegetal, Instituto de Biologia, Universidade Estadual de Campinas, 13083-875 Campinas, São Paulo, Brazil

- *Premise of the study:* We developed the first microsatellites for *Passiflora setacea* and characterized new sets of markers for *P. edulis* and *P. cincinnata*, enabling further genetic diversity studies to support the conservation and breeding of passion fruit species.
- *Methods and Results:* We developed 69 microsatellite markers and, in conjunction with assessments of cross-amplification using primers available from the literature, present 43 new polymorphic microsatellite loci for three species of *Passiflora*. The mean number of alleles per locus was 3.1, and the mean values of the expected and observed levels of heterozygosity were 0.406 and 0.322, respectively.
- *Conclusions:* These microsatellite markers will be valuable tools for investigating the genetic diversity and population structure of wild and commercial species of passion fruit (*Passiflora* spp.) and may be useful for developing conservation and improvement strategies by contributing to the understanding of the mating system and hybridization within the genus.

Key words: genetic diversity; genomic microsatellite-enriched library; molecular markers; *Passiflora*; simple sequence repeats; wild passion fruit.

The genus *Passiflora* L. (Passifloraceae) comprises approximately 400 species, of which at least 30% are distributed within Brazilian forests (Cervi et al., 2010). Species such as *P. edulis* Sims are important because of the economic value of their fruit (Faleiro et al., 2005). Certain wild species, including *P. setacea* DC. and *P. cincinnata* Mast., are of interest because of their potential use in genetic breeding. However, the limited number of molecular genetic diversity studies of this genus (Faleiro et al., 2005; Cerqueira-Silva et al., 2012) attests to the need for and relevance of novel molecular tools for studies of its populations and mating system.

Although diversity studies of passion fruit began in the late 1990s, efforts to use microsatellites only began in 2005 (Oliveira et al., 2005; Pádua et al., 2005), and studies related to the development of microsatellites have been published for *P. cincinnata* (Cerqueira-Silva et al., 2012) and *P. contracta* Vitta (Cazé et al., 2012) only recently. The markers available are still insufficient for performing consistent genetic studies of most *Passiflora* species because the evaluated populations exhibit low variability and percentages of polymorphic loci (between 0% and 26%) (Pereira, 2010; Ortiz et al., 2012; Cerqueira-Silva et al., 2012). Thus, considering the difficulty in obtaining informative microsatellites for *Passiflora* and to enhance the genetic investigation of both wild and commercial populations, we isolated, characterized, and evaluated the cross-amplifications of microsatellites for *P. edulis*, *P. setacea*, and *P. cincinnata*.

METHODS AND RESULTS

Two microsatellite-enriched genomic libraries were developed using genotypes from the germplasm collection of *P. edulis* (Pe-UESB01) and *P. setacea* (Ps-UESB01) from the Universidade Estadual do Sudoeste da Bahia (UESB; Itapetinga, Bahia, Brazil). Genomic DNA was isolated from fresh leaves using the cetyltrimethylammonium bromide (CTAB) method, and libraries were constructed following Billote et al. (1999). DNA samples (5 µg) were digested with *Afa*I and ligated to the double-stranded adapters 5′-CTCTTGCTTACGCGTGGACTA-3′ and 5′-TAGTCCACGCGTAAGCAAGAGCACA-3′. Enrichment was performed using a hybridization-based capture with $(GT)_8$ and $(CT)_8$ biotin-linked probes and streptavidin-coated magnetic beads (Streptavidin Magnesphere Paramagnetic Particles; Promega Corporation, Madison, Wisconsin, USA). The selected fragments were cloned into a pGEM-T Easy Vector (Promega Corporation) and used to transform *Escherichia coli* xl1-blue competent

[1]Manuscript received 19 July 2013; revision accepted 18 October 2013.

The authors thank the Conselho de Aperfeiçoamento de Pessoal de Nível Superior (CAPES, PROCAD-NF2008) for financial support, the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) for a research fellowship to A.P.S., and the Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) and CNPq for graduate fellowships to G.M.M. and J.G.P.V., respectively.

[7]Author for correspondence: anete@unicamp.br

doi:10.3732/apps.1300061

TABLE 1. Characteristics of the 69 new microsatellite markers developed for passion fruit species (17 markers for *Passiflora edulis* and 52 markers for *P. setacea*) and cross-amplification assays.

Locus	Primer sequences (5′–3′)	Allele size (bp)	Repeat motif	PCR amplification conditions[a]	GenBank accession no.	Cross-amplification		
						Pe	Ps	Pc
mPe-UNICAMP01	F: CCTGTCGGAAAGACTTCTGC R: GGATCGTTGTGGAGTGTGGT	230–232	$(AC)_4$	TD58	KF142650		232	232
mPe-UNICAMP02	F: TCGAGTGAGATTGGCAGTG R: TTGGCTTCGAGGAGAAGAA	165–171	$(GT)_8$	TD58	KF142651		161–163	163
mPe-UNICAMP03	F: ATAGGCATTTCACAACAGCAC R: AAGCATCCGTGAGACAGGT	261	$(AC)_8$	TD58	KF142652		261	261
mPe-UNICAMP04	F: GCTAACAAGCCCAAATCAAC R: CAGACCATGAGACGGCAGTA	296	$(CA)_5$	TD65	KF142653		296	296
mPe-UNICAMP05	F: CGGGGTTATGCAAGGTAACA R: ACTGGGTGGACTAGGAAACG	121	$(TG)_8$	TD65	KF142654		—	—
mPe-UNICAMP06	F: GTTCGAACCTTGGTTCTCTTG R: AATCCTCTCCCGGTATCCAC	292	$(TG)_4$	TD65	KF142655		—	290–320
mPe-UNICAMP07	F: GGAACCGTGTGATGGGATAC R: ACCGATTGACAGCTCTGCC	255	$(AG)_8$	TD65	KF142656		255	255
mPe-UNICAMP08	F: GCTGAGAACCCCGTGACTTA R: CGAGTATGGCACATCCCTG	196	$(CA)_4$	TD65	KF142657		196	196
mPe-UNICAMP09	F: TGCCTCTCGGATATTTACAGC R: CGCATGTCCCCATACGAC	212	$(AC)_5$	TD58	KF142658		212	248–261
mPe-UNICAMP10	F: GTCACTGCAGCCTGGTATAGTT R: GAACATATTCGGCAGATGGA	251	$(CT)_5$	TD58	KF142659		251	251
mPe-UNICAMP11	F: GCAGCAATCAATGCAATCAG R: GCCATTCTCCTCTCACCGTA	180	$(CA)_9(AT)_5$	TD58	KF142660		172	176
mPe-UNICAMP12	F: CACACAAGGCGTTTCTTACG R: TGATATGAACGATACGGTAGGC	214	$(CA)_7$	TD65	KF142661		—	—
mPe-UNICAMP13	F: TTCGTGCATTGTTCATTACC R: GCCTTCTTTGTCATGTTGGA	202	$(TC)_5$	TD58	KF142662		202	166–168
mPe-UNICAMP14	F: GACTTCGTATGACGCCAGGT R: TGCAAGAATCCGAAGACTCA	263	$(CA)_8$	TD65	KF142663		263	260
mPe-UNICAMP15	F: CATTCCTCACCCTCACGAA R: TGGTTGTGTGGTTTGTGCTT	253	$(AC)_5$	TD58	KF142664		253	253
mPe-UNICAMP16	F: CGTGGGTGAGTGTGAATGAG R: TGATGTGAGCATGGTTGGTT	195	$(AT)_4(TG)_{11}$	TD65	KF142665		—	—
mPe-UNICAMP17	F: GCCACGTGCAATGTCAGT R: CGTGCTGTGACCAAGGAG	300	$(AC)_9$	TD65	KF142666		—	—
mPs-UNICAMP01	F: TAGCTTAACACAATGCAACAGA R: CAACGGAGAACGATGTCAG	153–154	$(TG)_5(TG)_5$	TD58	KF171014	158–168		154
mPs-UNICAMP02	F: TAGCTTAACACAATGCAACAGA R: CAACGGAGAACGATGTCAG	154–156	$(TG)_5(TG)_5$	TD58	KF171015	160–170		156
mPs-UNICAMP03	F: GTAGCGTCTCGGCAGGTC R: ACTCTAAGTCGGCCACTCTTG	176–177	$(CT)_4$	TD65	KF171016	176		176
mPs-UNICAMP04	F: CAACAGGAGGTGAGGTGTGA R: GACAGTGCAACTTTAGGCGAC	156–157	$(TG)_4$	TD65	KF171017	156		156
mPs-UNICAMP05	F: TCGGTCTTCGTATTCAACTCTG R: GAGGAACTGGCATCGCAT	194–218	$(CT)_8$	61.5°C	KF171018	210–220		213–216
mPs-UNICAMP06	F: GTTGGATCAAAGGGTCACA R: CAACTACTGGATCGAACTGGTA	218–224	$(CGTG)_3(ATGA)_3$	TD65	KF171019	194–224		215
mPs-UNICAMP07	F: ACAGGGGTGAGGCACATTC R: TCTGTTATTATCATCGGCAGGA	143–145	$(CA)_4$	TD58	KF171020	—		—
mPs-UNICAMP08	F: AGTGCCAGTGGCTTCGTATT R: GATCGTCATGGCTGTTGCTA	207–211	$(TGCAA)_3$	TD65	KF171021	174		176
mPs-UNICAMP09	F: GGGCCGTTGTCAAAGTAGT R: GAGGTTAAGGCAAGCACTG	250–268	$(AC)_4$	61.5°C	KF171022	258–260		260
mPs-UNICAMP10	F: ACTCTCACCTCAATCGACC R: AATTGTTACTCCGTTTCTCTGA	256–260	$(AG)_4(GT)_5(GT)_4$	60°C	KF171023	264–268		260–268
mPs-UNICAMP11	F: CAGACGTTGTGTTTTGGTAAT R: TCAGGTTAGGAAGCTGCATC	232–270	$(CA)_4(CA)_4(AT)_4$	60°C	KF171024	262		—
mPs-UNICAMP12	F: ACAGGGGTGAGGCACATACA R: GTAGTGCGTGGCTTGGGTAG	201–204	$(CA)_4$	TD65	KF171025	208		208
mPs-UNICAMP13	F: CCTATACCTGCCCAGTCAGC R: ACTTAAGCACCCCAATCGTT	146–148	$(CA)_4$	TD65	KF171026	144		—
mPs-UNICAMP14	F: CGTTCATAAGTGAATCAGTCAA R: GGATCGACAAACAAAGGTAGA	112–116	$(CA)_4$	TD65	KF171027	114		114
mPs-UNICAMP15	F: TATGGAGTTGCGAGGCTTTAG R: CGGGCAACGAACACTTTATT	145–148	$(GT)_4$	60°C	KF171028	143–145		146
mPs-UNICAMP16	F: GAGAAAGCGAGTCAGCGAGA R: GACTCCAATATCGGCACTTCA	157–165	$(GAG)_6(CAA)_4$	TD65	KF171029	163–167		159–170

Table 1. Continued.

Locus	Primer sequences (5′–3′)	Allele size (bp)	Repeat motif	PCR amplification conditions[a]	GenBank accession no.	Cross-amplification		
						Pe	Ps	Pc
mPs-UNICAMP17	F: CATCCAACCTCCGAACCTTA R: TACCCAGTCCGGTCCATTAG	142–148	$(AC)_5$	60°C	KF171030	147		146
mPs-UNICAMP18	F: GGGGTTCTTCACTCATCCAC R: TGACGACTAGGGGATTCAGG	262–278	$(CA)_{10}(AT)_6$	TD65	KF171031	—		—
mPs-UNICAMP19	F: CTGTGGCAAGTGGCTAACAA R: CCACCCTACTCGACCAACTC	290–294	$(TG)_4$	60°C	KF171032	290		290
mPs-UNICAMP20	F: GCTGGCTCTAGCTCAACTCG R: GCCAGCATAGGATGTCAGGT	200	$(CT)_5$	TD65	KF171033	200		200
mPs-UNICAMP21	F: CCCAATCGCTGAGAGGAGT R: CGGTAGGCTCATTCGTGTCA	228	$(TG)_4$	TD58	KF171034	—		—
mPs-UNICAMP22	F: AGGCATGCCCATCAAATG R: CACTAAAACCTGCAAAGCGAA	131	$(GT)_5(GT)_4$	TD58	KF171035	—		—
mPs-UNICAMP23	F: GAGCAGCTAAAAGAAACCTAC R: TAGAGGTTGTGCTGGAGTC	298	$(AC)_5(CA)_4$	TD58	KF171036	298		298
mPs-UNICAMP24	F: GAGGTCCCACCAGTGTCAGT R: CTAGCGTCACCCTCCAGAAG	254	$(AG)_4$	TD58	KF171037	254		258–260
mPs-UNICAMP25	F: GTGTTTGTGGCGATGTGATTA R: GACAAACGTTGTTTCCGCTC	162	$(AAG)_5$	TD58	KF171038	162		162
mPs-UNICAMP26	F: TGTGGCATGTGTATGACTTGAT R: CATAGATATGGGATGAGCGACA	166	$(TG)_4$	TD58	KF171039	166		174
mPs-UNICAMP27	F: AGATGGAACAGGTGGGTGAG R: TAGGCTTGTTCTGGCTCTGG	151	$(CCA)_5$	TD58	KF171040	151		151
mPs-UNICAMP28	F: AATTGTCATCGGTAAACCTGC R: TGCCATTGCGAGTGAATAAG	274	$(AC)_6$	TD58	KF171041	274		274
mPs-UNICAMP29	F: GAGAAATCTCAGCACACGCA R: CGGTTCTTGGTTTTGTGGAT	204	$(CA)_5$	TD58	KF171042	—		—
mPs-UNICAMP30	F: CGGCTGAAGGAGGAGGTAG R: TGAAAAACAAGTCAGCCAACA	118	$(GT)_6$	TD58	KF171043	—		—
mPs-UNICAMP31	F: GGTGTGGTAGCCTGTTTGTC R: CCGCATCTCTTACATCGTTA	211	$(TG)_4(GT)_5$	TD65	KF171044	215		215–219
mPs-UNICAMP32	F: CAGACGTTGCATCTTGGTAAT R: CATCGGAGGAGTTTTACACATT	172	$(CA)_4(AC)_9(AT)_6$	TD65	KF171045	172		172
mPs-UNICAMP33	F: GCAGCAATCAATGCAATCAG R: GCCATTCTCCTCTCACCGTA	184	$(AT)_4(CA)_{10}(AT)_6$	TD65	KF171046	184		184
mPs-UNICAMP34	F: GGCAGGATATGCTTTGGTT R: GCTGTCGGACACATGGAC	162	$(TC)_{10}$	TD65	KF171047	160		158–161
mPs-UNICAMP35	F: TCGAGAGTTGCGTGTGTTTC R: CATTCTCCTGCCACCTGAGT	183	$(TG)_4$	TD65	KF171048	183		183
mPs-UNICAMP36	F: GGGAGTCGGGTTGAGTTA R: AGTCGAGGACCAGTCAAAG	228	$(TG)_4(TG)_7$	TD65	KF171049	228		228
mPs-UNICAMP37	F: TTGTTTGGGTTAGCGTGTGAG R: CCCTGCCACCTGAGTAATCA	172	$(TG)_6$	TD65	KF171050	172		172
mPs-UNICAMP38	F: CCTGACCTCTGGCACTACC R: GAGGCGTATCAGGCTTTGA	112	$(TGC)_6$	TD65	KF171051	112		112
mPs-UNICAMP39	F: GGAGGGTTGTTGTGTGAGTG R: CTCCTGTCGGAAAGACTTCTG	230	$(GT)_4$	TD65	KF171052	230		—
mPs-UNICAMP40	F: GAATCAATGGAACACAAGCA R: CCAGCCCACTAGACCACCT	224	$(AC)_5$	TD65	KF171053	234		230
mPs-UNICAMP41	F: CTTCAGTGCAGCCTTCCAT R: ATACCGATACTCGCCTTGATAG	168	$(GT)_4$	60°C	KF171054	168		170
mPs-UNICAMP42	F: AGTGCCAGTGGCTTCGTATT R: GATCGTCATGGCTGTTGCTA	174	$(TGCAA)_3$	61.5°C	KF171055	174		174
mPs-UNICAMP43	F: CTCAGTGAGGAATAAGCAATCA R: ATTTGGCATGCTGTTACGC	192	$(CA)_4$	61.5°C	KF171056	198		198
mPs-UNICAMP44	F: AGTCGTGCTTGTGTTGTTGAG R: CCACTGTTGAGGTCCAGATG	275	$(GATT)_3$	TD65	KF171057	280		275
mPs-UNICAMP45	F: CCTATACCTGCCCCAGTCAG R: GTATGTGTGTGCCGTGGATT	110	$(AT)_4(CA)_4$	TD65	KF171058	110		110
mPs-UNICAMP46	F: TGCGTGTTGTCCCACCAT R: GACTGAGCGGACTCACATCA	138	$(CT)_8$	TD65	KF171059	138		138–139
mPs-UNICAMP47	F: AAATTTCGGCATGGTTTATG R: CCGAGATCGTTGGAGCTTA	298	$(AC)_5(CA)_4$	60°C	KF171060	294		298
mPs-UNICAMP48	F: AGCTTACCGGCTCACTCTTG R: GACAGGCTTGGAACTGGAAT	144	$(AC)_6$	60°C	KF171061	143		142
mPs-UNICAMP49	F: TGTATGAGTGAGAATGAGCCCA R: CAATCAACATGAGACAAGCGG	118	$(TA)_4$	TD65	KF171062	126		126
mPs-UNICAMP50	F: TTCTGCGAAACTGGTGAGTG R: CGCCCGTATTTTGTCATGA	202	$(TA)_6$	60°C	KF171063	202		202

Table 1. Continued.

Locus	Primer sequences (5′–3′)	Allele size (bp)	Repeat motif	PCR amplification conditions[a]	GenBank accession no.	Cross-amplification		
						Pe	Ps	Pc
mPs-UNICAMP51	F: CTTGCACACTCACGGCTAAA R: CAACCTACTGGATCGAACTGAA	152	$(GT)_5$	60°C	KF171064	152		150–152
mPs-UNICAMP52	F: GTCCGTTGAGAACCCCGTA R: ACCAATCGTTGAGAGTTCGTG	118	$(AT)_5$	60°C	KF171065	118		—

Note: — = unsuccessful amplification; Pc = *Passiflora cincinnata*; Pe = *Passiflora edulis*; Ps = *Passiflora setacea*.

[a]TD65 and TD58 indicate touchdown PCR programs with temperatures ranging from 65°C to 55°C and 58°C to 48°C, respectively.

cells (Stratagene, La Jolla, California, USA). The recombinant colonies were selected using blue/white screening. In total, 480 positive clones (192 for *P. edulis* and 288 for *P. setacea*) were randomly selected and double-sequenced using an ABI PRISM 377 automated DNA sequencer (Applied Biosystems, Foster City, California, USA). Every sequence was aligned and edited using SeqMan software (DNASTAR, Madison, Wisconsin, USA). We used the MICROSAT software developed by A. M. Risterucci at the Centre de Coopération Internationale en Recherche Agronomique pour le Développement (CIRAD, France; unpublished) to identify and eliminate the adapters and restriction sites from the edited sequences.

Sequences containing microsatellites (134 for *P. edulis* and 114 for *P. setacea*) were identified using the SSR Identification Tool (SSRIT; Temnykh et al., 2001). Approximately 85% of the microsatellite motifs observed for both of the species were dinucleotides. We designed a total of 30 (*P. edulis*) and 75 (*P. setacea*) primer pairs using PrimerSelect (DNASTAR) and Primer3Plus (Untergasser et al., 2007). The 105 primer pairs exhibited the following characteristics: annealing temperatures ranging from 45°C to 65°C (with a maximum difference of 3°C between the forward and reverse primers), CG concentrations ranging from 40% to 70%, and amplified product sizes varying from 100 to 300 bp. We used 16 genotypes of passion fruit (eight for each species) for the amplification tests. PCRs were conducted using a final volume of 15 µL (containing 15 ng of template DNA) with the reagents and concentrations described by Cerqueira-Silva et al. (2012). Every marker was evaluated by PCR amplification as follows: 94°C for 5 min; 34 cycles of 94°C for 1 min, 60°C for 1 min, and 72°C for 1 min; and a final extension at 72°C for 10 min. The loci that showed unsatisfactory amplification with an annealing temperature of 60°C were subjected to two different touchdown PCR protocols (TD 65–55°C and TD 58–48°C) as follows: an initial denaturation (94°C for 5 min); 10 cycles of 94°C for 1 min and an annealing temperature decreasing by 1°C from 65–55°C or 58–48°C every cycle for 1 min; 14 cycles of 94°C for 1 min, 55°C or 48°C

Table 2. Results of the initial screening of polymorphic microsatellite markers in populations of *Passiflora edulis*, *P. setacea*, and *P. cincinnata*.

Locus	*P. edulis* (*N* = 42)				*P. setacea* (*N* = 42)				*P. cincinnata* (*N* = 31)			
	A	H_o	H_e	PIC	*A*	H_o	H_e	PIC	*A*	H_o	H_e	PIC
mPe-UNICAMP01	2	0.051	0.047	0.476	1	—	—	—	1	—	—	—
mPe-UNICAMP02	4	0.651	0.515	0.404	2	0.261	0.497	0.371[a]	1	—	—	—
mPe-UNICAMP06	1	—	—	—	0	—	—	—	2	0.083	0.079	0.077
mPe-UNICAMP09	1	—	—	—	1	—	—	—	6	0.458	0.679	0.628[a]
mPe-UNICAMP13	1	—	—	—	1	—	—	—	3	0.055	0.205	0.191[a,b]
mPs-UNICAMP01	5	0.578	0.723	0.642[a]	2	0.333	0.512	0.393[a]	1	—	—	—
mPs-UNICAMP02	5	0.631	0.768	0.704	2	0.333	0.511	0.389[a]	1	—	—	—
mPs-UNICAMP03	1	—	—	—	2	0.311	0.266	0.225	1	—	—	—
mPs-UNICAMP04	1	—	—	—	2	0.142	0.133	0.123	1	—	—	—
mPs-UNICAMP05	4	0.381	0.471	0.476[b]	4	0.261	0.593	0.468[a, b]	3	0.401	0.513	0.392
mPs-UNICAMP06	4	0.191	0.176	0.157	2	0.424	0.401	0.322	1	—	—	—
mPs-UNICAMP07	0	—	—	—	2	0.251	0.221	0.194	0	—	—	—
mPs-UNICAMP08	1	—	—	—	2	0.102	0.097	0.093	1	—	—	—
mPs-UNICAMP09	2	0.024	0.024	0.023	4	0.761	0.614	0.551	1	—	—	—
mPs-UNICAMP10	3	0.119	0.197	0.186[a]	3	0.357	0.583	0.493[a,b]	4	0.448	0.637	0.577[a]
mPs-UNICAMP11	1	—	—	—	3	0.166	0.157	0.149	0	—	—	—
mPs-UNICAMP12	1	—	—	—	2	0.208	0.187	0.371	1	—	—	—
mPs-UNICAMP13	1	—	—	—	2	0.282	0.456	0.351[a]	0	—	—	—
mPs-UNICAMP14	1	—	—	—	4	0.589	0.674	0.678[b]	1	—	—	—
mPs-UNICAMP15	2	0.024	0.024	0.023	3	0.101	0.531	0.411[a,b]	1	—	—	—
mPs-UNICAMP16	3	0.476	0.585	0.499	3	0.391	0.485	0.395	4	0.561	0.541	0.464
mPs-UNICAMP17	1	—	—	—	4	0.833	0.714	0.656[b]	1	—	—	—
mPs-UNICAMP18	0	—	—	—	4	0.524	0.454	0.412	0	—	—	—
mPs-UNICAMP19	1	—	—	—	2	0.189	0.173	0.566	1	—	—	—
mPs-UNICAMP24	1	—	—	—	1	—	—	—	2	0.125	0.187	0.169
mPs-UNICAMP31	1	—	—	—	1	—	—	—	3	0.217	0.326	0.282
mPs-UNICAMP34	0	—	—	—	1	—	—	—	5	0.401	0.671	0.592[a,b]
mPs-UNICAMP46	1	—	—	—	1	—	—	—	2	0.041	0.041	0.041
mPs-UNICAMP51	1	—	—	—	1	—	—	—	2	0.033	0.033	0.038
mPc-UNICAMP11[c]	4	0.237	0.447	0.424[b]	0	—	—	—	1	—	—	—
mPc-UNICAMP19[c]	0	—	—	—	4	0.418	0.411	0.367	1	—	—	—

Note: — = information not available; *A* = number of alleles per locus; H_e = expected heterozygosity; H_o = observed heterozygosity; PIC = polymorphism information content.

[a]Markers with the probability of null allele occurrence after a Bonferroni correction.

[b]Markers deviating from Hardy–Weinberg equilibrium after a Bonferroni correction ($P < 0.004$ [*P. edulis* and *P. cincinnata*]; $P < 0.002$ [*P. setacea*]).

[c]Microsatellite markers published by Cerqueira-Silva et al. (2012).

for 1 min, and 72°C for 1 min; and a final extension at 72°C for 10 min. For markers that showed inconsistent amplification after the touchdown protocols, we tested reactions with an annealing temperature gradient ranging from 65°C to 50°C. The products were visualized using vertical electrophoresis on 6% denaturing polyacrylamide gels run in 1× TBE and stained with silver nitrate. The product sizes were determined using a 10-bp DNA ladder (Invitrogen, Carlsbad, California, USA). In total, 17 and 52 markers generated consistent patterns of amplification that matched the expected sizes based on the sequenced fragments from *P. edulis* and *P. setacea*, respectively (Table 1). Cross-amplification assays were performed according to previously described protocols, with all 69 primer pairs showing a high percentage of amplification (88% [*P. edulis*], 70% [*P. setacea*], and 80% [*P. cincinnata*]) (Table 1). Cross-amplification assays were also performed with the 25 loci previously characterized for *P. cincinnata* (Cerqueira-Silva et al., 2012), presenting a percentage of amplification of 48% in *P. edulis* (mPc-UNICAMP02, -04, -06, -10, -11, -14, -15, -17, -18, -20, -21, and -24) and 28% in *P. setacea* (mPc-UNICAMP02, -04, -06, -10, -15, -19, and -20).

To characterize all the loci, we used genotypes from the germplasm collection of the Embrapa Mandioca Fruticultura Center (Empresa Brasileira de Pesquisa Agropecuária [EMBRAPA]), Cruz das Almas, Bahia, Brazil, and of the UESB, Itapetinga, Bahia, totaling 114 genotypes. For each species, 42, 42, and 30 genotypes from *P. edulis* (all from EMBRAPA), *P. setacea* (30 from EMBRAPA and 12 from UESB), and *P. cincinnata* (all from EMBRAPA), respectively, were used (Appendix S1). We performed a descriptive statistical analysis for all the polymorphic loci using GENEPOP software (Raymond and Rousset, 1995; Table 2). The polymorphism information content was calculated using PIC Calculator software (Kemp, 2002), and the probability of null alleles was estimated using MICRO-CHECKER software (van Oosterhout et al., 2004), with significant probabilities between two and six loci observed for the three species evaluated (Table 2).

The percentage of polymorphic microsatellites observed was 15% in *P. edulis*, 29% in *P. setacea*, and 20% in *P. cincinnata*, totaling 11, 21, and 11 polymorphic loci, respectively (Table 2). This low number of polymorphic loci was expected because low variability appears to be a characteristic of the genus *Passiflora*, as suggested by Cerqueira-Silva et al. (2012). The number of alleles per locus ranged from two to six, with a mean of 3.1 for the three species evaluated; overall, the observed heterozygosity was lower than expected heterozygosity. Of the 31 polymorphic microsatellites, only one (*P. edulis*), six (*P. setacea*), and two (*P. cincinnata*) showed significant deviation from Hardy–Weinberg equilibrium (HWE) after a Bonferroni correction. Deviations from HWE can be explained by linkage disequilibrium (LD) or the occurrence of null alleles. Among the 320 possible pairs of microsatellites, we observed significant LD for two pairs (in *P. edulis*; $P < 0.004$), 49 pairs (in *P. setacea*; $P < 0.002$), and one pair (in *P. cincinnata*; $P < 0.004$) after a Bonferroni correction. However, with no additional information, LD should not be attributed solely to physical linkages among loci because of the possibility of population processes such as nonrandom mating (Hedrick, 2005).

CONCLUSIONS

We present the first set of microsatellites developed for *P. setacea* and characterize new markers for *P. edulis* and *P. cincinnata*, thereby increasing the number of available markers for these species. This effort potentiates the use of microsatellites in genetic studies of wild and commercial populations of *Passiflora* species, enabling the development of more efficient conservation and genetic breeding strategies.

LITERATURE CITED

Billote, N., P. J. L. Lagoda, A. M. Risterucci, and F. C. Baurens. 1999. Microsatellite-enriched libraries: Applied methodology for the development of SSR markers in tropical crops. *Fruits* 54: 277–288.

Cazé, A. L. R., R. A. Kriedt, L. B. Beheregaray, S. L. Bonatto, and L. B. Freitas. 2012. Isolation and characterization of microsatellite markers for *Passiflora contracta. International Journal of Molecular Sciences* 13: 11343–11348.

Cerqueira-Silva, C. B. M., E. S. L. Santos, A. M. Souza, G. M. Mori, E. J. Oliveira, R. X. Corrêa, and A. P. Souza. 2012. Development and characterization of microsatellite markers for the wild South American *Passiflora cincinnata* (Passifloraceae). *American Journal of Botany* 99: e170–e172.

Cervi, A. C., M. A. Milward-De-Azevedo, and L. C. Bernacci. 2010. Passifloraceae. *In* Lista de Espécies da Flora do Brasil [online]. Jardim Botânico do Rio de Janeiro, Rio de Janeiro, Brazil. Website http://floradobrasil.jbrj.gov.br/jabot/floradobrasil/FB182 [accessed 19 January 2014].

Faleiro, F. G. F., N. T. V. Junqueira, and M. F. Braga. 2005. Germoplasma e melhoramento genético do maracujazeiro: Desafios da pesquisa, 55–78. *In* F. G. Faleiro, N. T. V. Junqueira, and M. F. Braga [eds.], Maracujá: Germoplasma e melhoramento genético. Embrapa Cerrados, Planaltina, Brazil.

Hedrick, P. W. 2005. Genetics of populations, 3rd ed. Jones Bartlett Publishers, Boston, Massachusetts, USA

Kemp, S. 2002. PIC Calculator Extra. Website http://www.genomics.liv.ac.uk/animal/pic.html [accessed 25 April 2012].

Oliveira, E. J., J. G. Padua, M. I. Zucchi, L. E. A. Camargo, M. H. P. Fungaro, and M. L. C. Vieira. 2005. Development and characterization of microsatellite markers from the yellow passion fruit (*Passiflora edulis* f. *flavicarpa*). *Molecular Ecology Notes* 5: 331–333.

Ortiz, D. C., A. Bohórquez, M. C. Duque, J. Tohme, D. Cuéllar, and T. M. Vásquez. 2012. Evaluating purple passion fruit (*Passiflora edulis* Sims f. *edulis*) genetic variability in individuals from commercial plantations in Colombia. *Genetic Resources and Crop Evolution* 59: 1089–1099.

Pádua, J. G., E. J. Oliveira, M. I. Zucchi, G. C. X. Oliveira, L. E. A. Camargo, and M. L. C. Vieira. 2005. Isolation and characterization of microsatellite markers from the sweet passion fruit (*Passiflora alata* Curtis Passifloraceae). *Molecular Ecology Notes* 5: 863–865.

Pereira, G. S. 2010. Desenvolvimento de Marcadores SSR, M-AFLP e SNP visando à integração de mapas genético-moleculares de *Passiflora alata* Curtis. M.Sc. dissertation, Universidade Estadual de São Paulo, Escola Superior de Agricultura 'Luiz de Queiroz', Piracicaba, São Paulo, Brazil.

Raymond, M., and F. Rousset. 1995. GENEPOP (version 1.2): Population genetics software for exact tests and ecumenicism. *Heredity* 86: 248–249.

Temnykh, S., G. Clerck, A. Lukashova, L. Lipovich, S. Cartinhour, and S. McCouch. 2001. Computational and experimental analysis of microsatellites in rice (*Oryza sativa* L.): Frequency, length variation, transposon associations, and genetic marker potential. *Genome Research* 11: 1441–1452.

Untergasser, A., H. Nijveen, X. Rao, T. Bisseling, R. Geurts, and J. A. M. Leunissen. 2007. Primer3Plus, an enhanced web interface to Primer3. *Nucleic Acids Research* 35 (Supplement 2): W71–W74.

van Oosterhout, C. V., W. F. Hutchinson, D. P. M. Wills, and P. Shipley. 2004. MICRO-CHECKER: Software for identifying and correcting genotyping errors in microsatellite data. *Molecular Ecology Notes* 4: 535–538.