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Applications in Plant Sciences

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Primer Note

Microsatellites for *Carpotroche brasiliensis* (Flacourtiaceae), a useful species for agroforestry and ecosystem conservation[1]

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- *Premise of the study:* We developed microsatellite markers for *Carpotroche brasiliensis* (Flacourtiaceae), a dioecious tree that is used as a food resource by midsize animals of the Brazilian fauna.
- *Methods and Results:* We designed 30 primer pairs using next-generation sequencing and classified 25 pairs as polymorphic. Observed heterozygosity ranged from 0.5 to 1.0, and expected heterozygosity ranged from 0.418 to 0.907. The combined probability of exclusion was greater than 0.999 and the combined probability of identity was less than 0.001, indicating that these microsatellites are appropriate for investigations of genetic structure, individual identification, and paternity testing.
- *Conclusions:* The developed molecular tools may contribute to future studies of population genetics, answering ecological and evolutionary questions regarding efficient conservation strategies for *C. brasiliensis*.

Key words: *Carpotroche brasiliensis*; endemic species; Flacourtiaceae; next-generation sequencing; simple sequence repeat (SSR); tropical forest.

Carpotroche brasiliensis (Raddi) A. Gray (Flacourtiaceae), an endemic species from the Brazilian Atlantic Forest and popularly known as sapucainha, produces nutritious fruits that are rich in medicinal substances. The fruits, which are high in fat and mineral residues (Pinto et al., 2012), are dispersed by important forest fauna such as paca (*Agouti paca*) and agouti (*Dasyprocta agouti*) (Zucaratto et al., 2010). The oil extracted from its seeds, known as chaulmoogra oil, is of high economic value because it is used for medicinal and cosmetic purposes. In Camamu-Maraú County, State of Bahia, Brazil, farmers grow *C. brasiliensis* and *Theobroma cacao* L. (cocoa) in the shade of native canopy tree species. Therefore, *C. brasiliensis* is an important economic agricultural crop in this agroforestry system. Furthermore, it is a valuable species for maintaining diversity and species richness in the agricultural-ecological landscape (Bhagwat et al., 2008).

In this study, we developed microsatellite (i.e., simple sequence repeat [SSR]) markers because they are considered to be the most polymorphic class of markers, and can usually be treated as neutral (Tautz, 1989; Weber, 1990). As a result, they are often useful for population genetic studies. The advent of next-generation sequencing (NGS) allows whole genomes to be mined very efficiently to identify SSRs. Here, we report on the first set of microsatellites for *C. brasiliensis*, which is important for understanding the genetic behavior for breeding purposes of this commercially and ecologically valuable species.

METHODS AND RESULTS

Fresh *C. brasiliensis* leaves were collected from two populations (Pau Coco and Santa Rita) in Camamu-Maraú County, State of Bahia, Brazil. To design and characterize the developed markers, we sampled a total of 32 trees (16 individuals from each population). Sampling coordinates, voucher numbers, and Universidade Estadual de Santa Cruz (UESC) DNA Bank codes are provided in Appendix 1. The DNA was isolated using the cetyltrimethylammonium bromide (CTAB) extraction protocol (Doyle and Doyle, 1990). The company ecogenics GmbH (Zurich, Switzerland) enriched libraries for AG and AC motifs and performed next-generation sequencing (Roche 454 pyrosequencing platform [454 Life Sciences, a Roche Company, Branford, Connecticut, USA]) with high-quality DNA that was extracted from the leaves of a single specimen. After sequencing, we designed microsatellite primers using the software Primer3 (Rozen and Skaletsky, 1999).

We performed PCR using 7.5 ng of genomic DNA, 1.3 µL of 10× buffer (10 mM Tris [pH 9.0], 50 mM KCl, and 1.5 mM $MgCl_2$), 20 mM $MgCl_2$, 3.25 mM each dNTP, 3.6 mg bovine serum albumin (BSA), 1 unit of *Taq* DNA polymerase (Phoneutria, Belo Horizonte, Minas Gerais, Brazil), 3.9 mM primers (forward, reverse, and M13 tail [CACGACGTTGTAAAACGA]), and 1.43 mM tail-complementary primer labeled with fluorochromes (6-FAM, VIC, PET, or NED [Applied Biosystems, Foster City, California, USA]). The amplification reactions were carried out under the following conditions: an initial step at 94°C for 5 min; followed by 30 cycles of 94°C for 45 s, annealing temperature (54–58°C, see Table 1 for details of each primer) for 45 s, and 72°C for 1 min; followed by eight cycles of 72°C for 1 min, 53°C for 1 min, and 72°C for 1 min; with a final extension step at 72°C for 10 min. In this study, all of the amplification reactions were verified on 1.5% agarose gels before capillary

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TABLE 1. Characteristics of 30 *Carpotroche brasiliensis* microsatellite loci.

Locus	Primer sequences (5′–3′)	Repeat motif	Allele size range (bp)	T_a (°C)	GenBank accession no.
CA_1	F: *GGAGTTCCCTACTCCAAATGAG R: GTTGCACTCGTATGGTTTCATC	$(TG)_{13}$	234–272	55	KM096783
CA_2	F: *TACGTGAGTTTTTGGAGGCG R: TGTAGCTGGTCTCGTTGGAC	$(TGA)_{19}$	163–204	58	KM096784
CA_3	F: *CCATGGACTGTGAGGGAGAG R: AGCTTGACTGGGATAGTCGC	$(CA)_{11}$	224–264	58	KM096785
CA_7	F: *CGCATACACTCACACCCAC R: CGATCAAGACAAGCCCCAAC	$(CT)_{11}$	164–194	58	KM096786
CA_8	F: *ACAAAGAGTGGAAGCAGAGG R: TCCCAACTTGTCCCCTTTCG	$(AG)_{14}$	160	56	KM096787
CA_9	F: *AATGCCTCTACATATAATCATGCC R: GCTGGAAGGGTTTAGGTTGATAAG	$(AC)_{17}$	166–192	56	KM096788
CA_10	F: *TCTCCAATCCCCACTTGCTC R: ATCATGAATTAAGATTTCCTTATACGC	$(TG)_{13}$	198–232	56	KM096789
CA_12	F: *AGACCCACAGTCCTGTCTTC R: CAGAGGGCGTGTATCTAGGC	$(AC)_{13}$	148–178	56	KM096790
CA_13	F: *TGCTCACATGGCTTGGTAATC R: AATCATGCATACAAGGAAGCC	$(CA)_{19}$	166–208	56	KM096791
CA_15	F: *AGGAAGATGCTCAAGGCAAG R: CTTCTGCAAGTTGGCCACAG	$(AC)_{11}$	220–238	56	KM096792
CA_16	F: *GTTGGTGTTCCTGGACTTGC R: GGTGCTGTTGTTTCTTGCAC	$(AC)_{14}$	154–208	54	KM096793
CA_18	F: *TCACTATCTATTATCCGTTGGAGC R: CGTGTGGCGATATATAAATGAAATTAG	$(AC)_{19}$	150–186	54	KM096794
CA_19	F: *GATGAACTGCCACCAAGCTC R: TGTGCATCTAGCTACTGTTTGTC	$(CA)_{12}$	213	55	KM096795
CA_20	F: *AAGGTGGTCCAAAACGATGC R: CTGCTCTTCGTGACGGTATTG	$(AG)_{16}$	188–218	56	KM096796
CA_21	F: *AGAGGTCTCAGTTTAACAGTCTC R: ATAGCCCAGACCTACATGGC	$(AAT)_{9}$	161–221	58	KM096797
CA_23	F: *ACGAAGAGGGAGTGAAAATGAC R: CGATTGCGAGCGAGGATAC	$(AAT)_{15}$	166–217	54	KM096798
CA_25	F: *CCCACTAACGTTTTGTTTGGTG R: GGGTAACCATGCACTTCTATGC	$(AC)_{15}$	164–192	58	KM096799
CA_26	F: *TCTTGCTGTTTCAATAGTGGAC R: TCGACCAAAAAGTTAACACCTC	$(GT)_{11}$	144–196	56	KM096800
CA_27	F: *TGGCTTCAGACCAGAGCTTC R: GATAGGGCACAATTGGCGTC	$(CAA)_{8}$	146	56	KM096801
CA_29	F: *TTATGGAGCTGTGGTGGAGC R: AGAAGAACAATAGCACCAGTAGC	$(TTG)_{11}$	247–268	56	KM096802
CA_30	F: *TGCATACAAGTCCCCATCAAAG R: AGCTTGAGGAAAGCTGTGTC	$(CA)_{16}$	206–222	56	KM096803
CA_32	F: *AAGCTGATTTCCGGCCAAAC R: ATGCTTGCATTGGTGGCTC	$(TC)_{11}$	142–202	58	KM096804
CA_34	F: *AGAACATAATCAGAGCGTTAGGG R: AGTGATGCGGTCAGTTATCC	$(AC)_{14}$	180–194	56	KM096805
CA_35	F: *AAAGAAGGTTGGTGGCAACG R: AGCAATGTGGGACGTGATTG	$(TC)_{11}$	258–272	56	KM096806
CA_36	F: *TCCACCTGCAATAATATCTGGG R: CCCCAACTGCCCAAATTCC	$(GA)_{18}$	158–216	56	KM096807
CA_38	F: *TCTTGCCTCTTACGCACATC R: TATGACAGGCGACCTAGCAC	$(CA)_{17}$	186	56	KM096808
CA_39	F: *TGAACCTGAATAGTTGAACCCC R: ACATTGTTTTATTACCAGTTGGCTTG	$(GA)_{19}$	142–172	58	KM096809
CA_40	F: *TGCTTTGTTGTATATCTTGGGGC R: ACAAGCAAGGAGTGGAGGTC	$(AC)_{13}$	224–258	57	KM096810
CA_41	F: *ACACTCATCCAACCACCAGC R: CTGTGTGGTCCATGGGATATG	$(CA)_{11}$	116–126	54	KM096811
CA_42	F: *GCACCACCTGTGTCATTCTC R: GAGCAGCGTCAGGTCAAAAG	$(GA)_{11}$	239	55	KM096812

Note: T_a = annealing temperature.
*M13 tail (CACGACGTTGTAAAACGA) added.

electrophoresis, which was performed on an ABI 3500 Genetic Analyzer (Applied Biosystems).

SSR allele peaks were detected using GeneMarker version 1.95 (SoftGenetics, State College, Pennsylvania, USA). The number of alleles (A), observed heterozygosity (H_o), expected heterozygosity (H_e), fixation index (F), and gametic disequilibrium were calculated using FSTAT version 2.9.3.2 (Goudet, 1995). The combined probability of exclusion (P_e) and combined probability of identity (P_{ID}) were calculated using CERVUS 3.0.3 (Marshall et al., 1998). Departure from Hardy–Weinberg equilibrium (HWE) was estimated using GenAlEx 6.5 (Peakall and Smouse, 2012).

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TABLE 2. Number of designed primers and next-generation sequencing results for *Carpotroche brasiliensis* library synthesis.

Category	Number
Total no. of sequences examined	380
No. of primer sequences found	127
No. of dinucleotide repeat motifs	26
No. of trinucleotide repeat motifs	18
No. of designed primers	30
No. of polymorphic loci	25
Maximum amplicon size	250
Minimum amplicon size	96
Maximum repeat length	19
Minimum repeat length	7

A total of 380 sequences were generated, of which 33.4% had microsatellites. Thirty primer pairs were designed, 25 of which were polymorphic, with expected fragment sizes and patterns of amplification. NGS results, including the number of designed primers, information about amplicon size, and repeat length, are available in Table 2. The description of primers and characterization of each locus are provided in Table 1. Six types of repeat motifs were identified in the primer set. The dinucleotide motif AC/TG had the highest percentage of occurrence, followed by AG/TC (Fig. 1); this indicates that, unexpectedly (Morgante and Olivieri, 1993), the AG motif seems to be the second most common in the *C. brasiliensis* genome.

The 25 studied loci had a maximum of 12 alleles per locus. Estimates of H_o consistently exceed those of H_e, indicating an excess of heterozygotes as shown by a negative fixation index. We found significant differences in number (193 alleles in the Pau Coco population and 174 alleles in the Santa Rita population) and frequency of alleles between the analyzed populations. Additionally, we discovered private alleles in both populations (Fig. 2). For CA_3 and CA_21 (Fig. 2B and 2D), all of the alleles from the Santa Rita population were different from those that were found in the Pau Coco population. The allele frequency distribution was different for most of the loci. The Pau Coco population showed deviation from HWE in four loci (CA_1, CA_13, CA_21, and CA_32). In contrast, in the Santa Rita population, we observed deviations from HWE for three SSR loci (CA_7, CA_10, and CA_12) (Table 3). We did not observe gametic disequilibrium between pairs of loci, indicating that the entire set can be used for population and quantitative genetic studies. The average combined P_e was greater than 0.999, valorizing the developed molecular markers as well as the values obtained for the combined P_{ID} (1.4×10^{-29} and 7.3×10^{-27} for the Pau Coco and Santa Rita populations, respectively) (Table 3).

CONCLUSIONS

The economic and ecological interest in *C. brasiliensis* makes studies related to population and quantitative genetics imperative for future advances in conservation and breeding programs for this species. The genetic characterization of these 25 new microsatellite loci indicates the accuracy of these new genetic tools for paternity tests, which can be used in studies of gene flow. Our results based on the distribution of allelic frequencies highlight the importance of selecting the best loci for each analytical purpose. For example, in ongoing research we are comparing the distance of gene flow occurring in natural and crop populations (agroforestry) to propose a management design to maximize the admixture between these populations. The planned distribution of *C. brasiliensis* plants in an agroforestry population will help both in the conservation of local fauna and in genetic diversity, which is important to start a breeding program for this species. Additionally, the microsatellite primers developed here can be applied to investigations of genetic structure and can also be transferred to other *Carpotroche* species. In short, these molecular markers will be useful for improving the knowledge of this important forest resource, which provides food for midsize fauna and economic support for families reliant on farming in southern Bahia, Brazil.

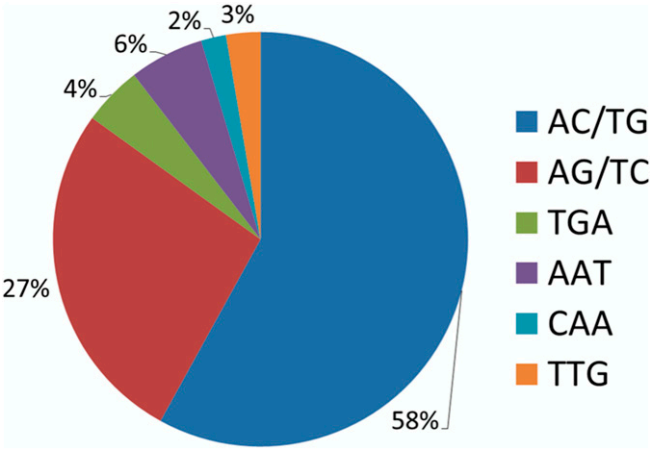


Fig. 1. Percentage of microsatellite motifs found in the *Carpotroche brasiliensis* genome.

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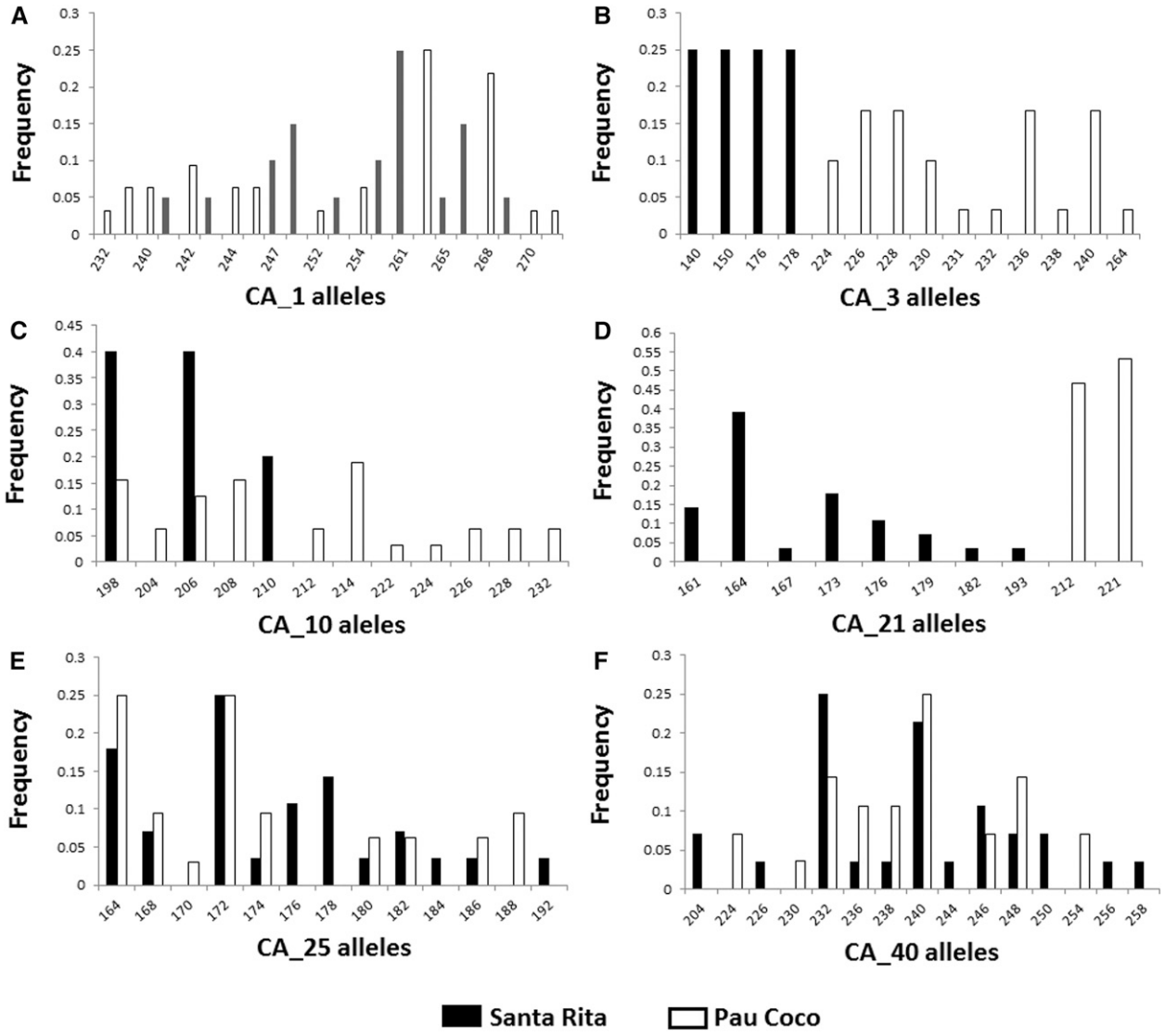


Fig. 2. Allele frequencies and distribution for the six most informative microsatellites (A–F) in the *Carpotroche brasiliensis* populations used in this study.

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TABLE 3. Genetic diversity statistics of 25 polymorphic *Carpotroche brasiliensis* microsatellite loci.[a]

	Pau Coco (n = 16)						Santa Rita (n = 16)					
Locus	A	H_o	H_e	P_e	P_{ID}	F	A	H_o	H_e	P_e	P_{ID}	F
CA_1	12	0.625	0.885[dhw]	0.110	0.034	0.152	10	1.000	0.905	0.112	0.034	−0.080
CA_2	8	0.875	0.841	0.183	0.059	−0.034	6	0.786	0.765	0.302	0.109	−0.030
CA_3	10	0.933	0.894	0.113	0.033	−0.039	4	0.917	0.757	0.348	0.124	−0.135
CA_7	8	0.938	0.857	0.162	0.051	−0.069	9	0.917	0.801[dhw]	0.213	0.077	−0.103
CA_9	6	0.938	0.645	0.462	0.205	−0.228	8	0.571	0.778	0.245	0.090	0.122
CA_10	11	0.875	0.907	0.091	0.027	−0.000	3	0	0.674[dhw]	0.505	0.206	1.000
CA_12	9	0.875	0.877	0.128	0.039	−0.013	9	0.800	0.885[dhw]	0.125	0.038	0.028
CA_13	7	0.938	0.806[dhw]	0.226	0.077	−0.111	6	0.938	0.744	0.326	0.122	−0.160
CA_15	7	1.000	0.849	0.179	0.056	−0.100	10	0.923	0.889	0.117	0.036	−0.037
CA_16	8	0.938	0.825	0.195	0.065	−0.084	7	0.692	0.849	0.183	0.058	0.075
CA_18	10	0.933	0.876	0.135	0.042	−0.054	6	0.727	0.762	0.308	0.115	−0.025
CA_20	11	0.875	0.851	0.152	0.050	−0.032	8	0.615	0.858	0.167	0.053	0.147
CA_21	2	0.938	0.514[dhw]	0.719	0.376	−0.306	8	0.929	0.802	0.221	0.078	−0.101
CA_23	7	1.000	0.762	0.290	0.108	−0.168	3	0	0.693	0.486	0.189	1.000
CA_25	9	0.938	0.863	0.145	0.046	−0.060	11	0.929	0.889	0.115	0.035	−0.039
CA_26	8	0.867	0.848	0.170	0.054	−0.040	3	0.250	0.419	0.651	0.388	0.355
CA_29	6	1.000	0.792	0.246	0.085	−0.148	6	0.786	0.852	0.185	0.057	0.019
CA_30	4	0.563	0.464	0.599	0.339	−0.147	4	0.714	0.548	0.545	0.272	−0.189
CA_32	6	1.000	0.792[dhw]	0.258	0.089	−0.142	11	0.929	0.839	0.164	0.056	−0.075
CA_34	6	1.000	0.800	0.255	0.086	−0.133	5	1.000	0.795	0.296	0.102	−0.145
CA_35	7	1.000	0.867	0.152	0.046	−0.088	6	0.875	0.704	0.344	0.138	−0.148
CA_36	8	0.688	0.829	0.188	0.062	0.0812	7	1.000	0.837	0.199	0.065	−0.115
CA_39	11	0.813	0.786	0.205	0.076	−0.078	8	0.846	0.766	0.244	0.093	−0.112
CA_40	9	1.000	0.889	0.118	0.036	−0.081	12	1.000	0.889	0.112	0.035	−0.083
CA_41	3	0.875	0.671	0.492	0.195	−0.157	4	0.500	0.426	0.628	0.378	−0.130
Mean	193*	0.897	0.799	0.999**	1.4×10^{-29}	−0.083	174*	0.745	0.765	0.999**	7.3×10^{-27}	0.042

Note: A = number of alleles; F = fixation index; H_e = expected heterozygosity; H_o = observed heterozygosity; n = sample size; P_e = combined probability of exclusion; P_{ID} = probability of identity.

[a] Geographic coordinates for the populations: Pau Coco 14°06′28.8″S, 93°14′58.1″W; Santa Rita 14°08′25.4″S, 93°17′38.7″W.

* Sum of the values.

** Combined values.

[dhw] Deviation from Hardy–Weinberg equilibrium ($P < 0.05$).

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Appendix 1. Voucher and location information of *Carpotroche brasiliensis* samples used in the current study. Voucher specimens are deposited at the herbarium of Universidade Estadual de Santa Cruz (UESC), Ilhéus, Brazil.

Population	Sample ID no.	Latitude	Longitude	DNA Bank code	Voucher no.
Pau Coco	1	14°08′58″S	39°14′25″W	BDU_621	RH-Uesc 20315
Pau Coco	2	14°08′58″S	39°14′25″W	BDU_622	
Pau Coco	3	14°08′59″S	39°14′24″W	BDU_623	
Pau Coco	4	14°08′58″S	39°14′24″W	BDU_624	
Pau Coco	5	14°08′59″S	39°14′24″W	BDU_625	
Pau Coco	6	14°08′58″S	39°14′24″W	BDU_626	
Pau Coco	7	14°08′59″S	39°14′24″W	BDU_627	
Pau Coco	8	14°08′59″S	39°14′24″W	BDU_628	
Pau Coco	9	14°08′59″S	39°14′24″W	BDU_629	
Pau Coco	10	14°08′59″S	39°14′24″W	BDU_630	
Pau Coco	11	14°08′59″S	39°14′25″W	BDU_631	
Pau Coco	12	14°08′59″S	39°14′25″W	BDU_632	
Pau Coco	13	14°08′59″S	39°14′25″W	BDU_633	
Pau Coco	14	14°08′59″S	39°14′25″W	BDU_634	
Pau Coco	15	14°08′59″S	39°14′25″W	BDU_635	
Pau Coco	16	14°08′59″S	39°14′25″W	BDU_636	
Santa Rita	1	14°08′16″S	39°17′51″W	BDU_661	RH-Uesc 20316
Santa Rita	2	14°08′31″S	39°17′41″W	BDU_662	
Santa Rita	3	14°08′30″S	39°17′41″W	BDU_663	
Santa Rita	4	14°08′31″S	39°17′41″W	BDU_664	
Santa Rita	5	14°08′36″S	39°17′42″W	BDU_665	
Santa Rita	6	14°08′36″S	39°17′43″W	BDU_666	
Santa Rita	7	14°08′37″S	39°17′43″W	BDU_667	
Santa Rita	8	14°08′37″S	39°17′44″W	BDU_668	
Santa Rita	9	14°08′39″S	39°17′44″W	BDU_669	
Santa Rita	10	14°08′42″S	39°17′45″W	BDU_670	
Santa Rita	11	14°08′42″S	39°17′45″W	BDU_671	
Santa Rita	12	14°08′42″S	39°17′45″W	BDU_672	
Santa Rita	13	14°08′43″S	39°17′45″W	BDU_673	
Santa Rita	14	14°08′32″S	39°17′39″W	BDU_674	
Santa Rita	15	14°08′31″S	39°17′39″W	BDU_675	
Santa Rita	16	14°08′16″S	39°17′51″W	BDU_676	

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