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Primer Note

Development of microsatellite markers in *Cratylia mollis* and their transferability to *C. argentea* (Fabaceae)[1]

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- *Premise of the study:* This work aimed to develop microsatellite markers for *Cratylia mollis* as tools to assess its genetic diversity and structure and to evaluate their potential cross-amplification in related species.
- *Methods and Results:* Microsatellite markers were developed using a microsatellite-enriched library and an intersimple sequence repeat library. From a set of 19 markers, 12 microsatellite loci were polymorphic and presented considerable variation in allele number (2–11), expected heterozygosity (0.226–0.883), and polymorphism information content per locus (0.212–0.870). Cross-amplification in *C. argentea* was successful in 16 loci, 12 of which were polymorphic (2–10 alleles).
- *Conclusions:* The polymorphism of this set of microsatellite markers for *C. mollis*, as well as their successful cross-amplification in *C. intermedia* and *C. bahiensis* and their transferability to *C. argentea*, supports their use in future comparative studies to understand the mechanism involved in population divergence and speciation in the genus.

Key words: *Cratylia*; cross-amplification; Fabaceae; microsatellite; population divergence; transferability.

Cratylia (Desv.) Kuntze is a South American genus distributed eastward from the Andes and southward from the Amazon River Basin. Five species are currently accepted: *C. argentea* (Desv.) Kuntze, *C. bahiensis* L. P. Queiroz, *C. hypargyrea* Mart. ex Benth., *C. intermedia* (Hassl.) L. P. Queiroz & R. Monteiro, and *C. mollis* Mart. ex Benth. (Queiroz and Coradin, 1995). *Cratylia argentea* is the most widespread, occurring from western Peru to Bolivia and Brazil in habitats including cerrado and seasonally dry forests. The other species are restricted to a particular type of vegetation: *C. hypargyrea* is found in Brazilian coastal Atlantic Forest, *C. bahiensis* and *C. mollis* grow in seasonally dry forest of northeastern Brazil, and *C. intermedia* occurs to the west of Paraná and to the northeast of Misiones Province in Argentina (Queiroz and Coradin, 1995).

Cratylia mollis and *C. argentea* are multipurpose shrub legumes with high potential for animal nutrition due to their nutritive value, particularly for dry season supplementation and silage (Argel and Lascano, 1998), and also show also tolerance to drought and adaptation to acidic soils (Andersson et al., 2006). Recent studies revealed that *C. mollis* seeds are an important lectin source (known as Cramoll) with applications for medicine (Fernandes et al., 2010). In the current study, our aim was to develop microsatellite markers to assess the genetic variation of *C. mollis*, and to assess their potential transferability to *C. argentea* and cross-amplification in other species of the genus.

METHODS AND RESULTS

Genomic DNA was isolated from one individual cultivated on the campus of the Universidade Estadual de Feira de Santana (*J. G. Rando 1257*, see Appendix 1) and extracted using the cetyltrimethylammonium bromide (CTAB) 2× protocol. Microsatellite markers were developed using both microsatellite-enriched library and intersimple sequence repeat (ISSR)–based cloning methods. A microsatellite-enriched library was built using the biotin-labeled microsatellite oligoprobes $(GT)_8$ and $(CT)_8$ and streptavidin-coated magnetic beads (Promega Corporation, Madison, Wisconsin, USA) according to Billotte et al. (1999). For the ISSR method, we performed PCR reactions with the ISSR primers GGCC$(AG)_8$, GGCC$(AC)_8$, CCGG$(AG)_8$, CCGG$(AC)_8$, GCGC$(AG)_8$, and GCGC$(AC)_8$ following closely the protocol of Provan and Wilson (2007). The selected fragments obtained by both methods were linked into pGEM-T Easy Vector (Promega Corporation, São Paulo, Brazil) and then transformed and cloned in TOP10 competent cells (Invitrogen, Life Technologies, Vila Guarani, São Paulo, Brazil).

Positive clones were amplified with the T7 (5′-TAATACGACTCACTATAGGG-3′) and SP6 (5′-ATTTAGGTGACACTATAGAA-3′) primers using the following conditions: denaturation step of 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 15 s, annealing at 55°C for 35 s, and extension at 72°C for 90 s; final extension was at 72°C for 7 min. DNA sequencing was performed with Big Dye Terminator Cycle Sequencing Kit (Applied Biosystems, São Paulo, Brazil). The software Imperfect Microsatellite Extractor (Mudunuri and Nagarajaram, 2007) was used for identification of simple sequence repeats in the nonredundant sequences.

Primers were designed using Primer3Plus (Rozen and Skaletsky, 2000) using the following criteria: product size range 100–300 bp, primer melting temperature (T_m) 50–60°C, primer GC 40–60%, maximum T_m difference: 2°C. Genotyping reactions were carried out in 10 µL with the Top Taq Master Mix kit (QIAGEN, Hilden, Germany) with approximately 4 ng of genomic DNA,

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0.15 µM of the forward primer (added M13 sequence: 5′-CACGACGTTGTAAAACGAC-3′), 0.30 µM of the reverse primer, and 0.60 µM of an M13-labeled tail (with one of four fluorescent dyes: 6-FAM, VIC, NED, or PET). For each primer pair, the optimal annealing temperature was established in gradient PCRs from 61–45°C; however, a touchdown program (60–54°C) was needed in some cases. For polymorphism assessment, individuals of two *C. mollis* populations (CMPOP_1, $N = 24$; CMPOP_2, $N = 25$) and 20 individuals cultivated from seeds of CMPOP_2 were genotyped. To test the cross-amplification and potential transferability, we sampled individuals obtained from the Laboratory of Plant Molecular Systematics (LAMOL) DNA bank at the Universidade Estadual de Feira de Santana (see Appendix 1): *C. argentea* ($N = 9$), *C. bahiensis* ($N = 2$), *C. hypargyrea* ($N = 2$), and *C. intermedia* ($N = 2$). Microsatellite profiles were analyzed with GeneMapper 4.0 (Applied Biosystems). The estimates of allelic diversity and deviations from Hardy–Weinberg equilibrium were carried out using GenAlEx 6.5 (Peakall and Smouse, 2006).

We amplified and sequenced 180 of 192 positive clones derived from the microsatellite-enriched and ISSR libraries and found 78 nonredundant sequences containing microsatellite loci. Percentage of dinucleotide motifs was higher (62%) than trinucleotide motifs (34%) or tetra-, penta-, and octanucleotide repeats (4% combined). The number of repetitions in dinucleotides varied from three to nine, in trinucleotides from three to five, in tetranucleotides from three to four, and in pentanucleotides from four to five. The motifs found in the sequences of the enriched library had fewer repetitions (3–4) than the ISSR library, and for this reason most of these were not used to design primers. Therefore, 32 primers pairs were designed (30 corresponding to the ISSR library), from which 19 presented amplification patterns compatible with the expected fragment sizes (Table 1).

Twelve microsatellite loci were polymorphic and presented considerable variation in allele number (2–11), observed heterozygosity (0.040–0.792), expected heterozygosity (0.226–0.883), and polymorphic information content per locus (0.212–0.870). The markers that were monomorphic in CMPOP_1 (CM_7, CM_15, CM_20) exhibited polymorphism in CMPOP_2 and vice-versa (CM_10), reflecting population differentiation in this species. Cross-amplification in *C. argentea* was successful in 16 loci; 12 of these were polymorphic, displaying an allele range of 2–4 in only nine individuals evaluated. Four monomorphic loci in *C. mollis* showed polymorphism in *C. argentea*. Cross-amplification success rates were also high in *C. intermedia* (15 loci) and *C. bahiensis* (12 loci); however, in *C. hypargyrea* only two microsatellite loci were successfully amplified (Table 2). Significant values ($P < 0.01$) of deviation from Hardy–Weinberg equilibrium were found in two loci of CMPOP_1 and one locus of CMPOP_2, where levels of observed heterozygosity were lower than expected (Table 2).

This heterozygote deficiency could reflect patterns of the population distribution and reproductive biology of *C. mollis*. This species presents a facultative breeding system, and experimental tests showed similar success in allogamous and autogamous crosses (Queiroz et al., 1997). It is pollinated by the carpenter bees *Xylocopa grisescens* Lepeletier and *X. cearensis* Ducke, which visit flowers that are simultaneously open in the same inflorescence before moving to other inflorescences, thus promoting geitonogamous crosses (L. P. Queiroz, unpublished observations). In addition, the species forms patchy populations on sandy soils (L. P. Queiroz, personal communication), which together with the pollinator behavior favors autogamy and low heterozygosity.

Table 1. Characterization of 19 microsatellite markers developed for *Cratylia mollis.*

Locus	Primer sequences (5′–3′)	Repeat motif	Product size (bp)[a]	T_a (°C)	M13 5′ Pigtail	GenBank accession no.
CM_1F	CAAGAAAGTGACCTAAATGG	$(TC)_2AC(TC)_2$	210–224	56	PET	KC351492
CM_1R	CTGATTCAGCTAAGGTAGACA					
CM_4F	CGTAGGAAGCACATGGTGTA	$(GTGCT)_2TGCT$	121	56	6-FAM	KC351493
CM_4R	CCAGAGCCACAAGGATAGAT					
CM_5F	CCTTGGAAAAGCTGAAGAGAAA	$(AG)_3AA(AG)_3$	136–142	60	VIC	KC351494
CM_5R	ATGGCCTCCTTCACATGC					
CM_6F	GGAGGCCATCTTTGAAGC	$(AGG)_3$	130–138	58–60	6-FAM	KC351494
CM_6R	GATGGTGGCTGTGATGGT					
CM_7F	GCTCAAGAGATTGTGAATGTG	$(AG)_4N_{12}(AGA)_3A(AGA)$	112–120	59	6-FAM	KC351495
CM_7R	CCTTCTACCTCACTCACTGCT					
CM_8F	GCAGTGAGTGAGGTAGAAGGAA	$(AATTA)_4(TTAAAAA)_4$	180–228	TD 60–54	NED	KC351495
CM_8R	CGAGGAAAACAAAACCCAAA					
CM_9F	CAATGGAAGACTTGGGGTTT	$(GA)_4$	170–180	58–60	VIC	KC351496
CM_9R	GGTTGGGCCTCTAATCTCCT					
CM_10F	GTTACTGGGTTCACTTTGGT	$(AGA)_3GGAAGA$	167–173	TD 60–54	VIC	KC351497
CM_10R	CTTCAACTTCTGGTTAATGG					
CM_11F	CATCATTAACATTGGACGGAAG	$(TG)_9$	186–212	59	NED	KC351499
CM_11R	CAACCAACACTCTCCACCTG					
CM_15F	CACGGTGCTCTGAAAGTTGT	$(CT)_6$	149–183	58	VIC	KC351502
CM_15R	AGAAGAGGGGAATGGGTTGT					
CM_18F	GCGTGAAGTATTGTATCAAG	$(ATA)_3ATT$	304–336	58–60	PET	KC351504
CM_18R	CAGTCACTACAAGTGCAGTAT					
CM_19F	CACGGGAGACAAGAGCGTA	$(GT)_4$	226–236	58–60	PET	KC351505
CM_19R	GGTCGCTGTACAATGAACCA					
CM_20F	CCTTGGAAAAGCTGAAGAGAAA	$(AG)_3AA(AG)_3$	246–258	56	PET	KC351506
CM_20R	CTTCAAGATGGTGGCTGTGA					
CM_21F	CAAGGTATGGGTAGGACAAGG	$(AG)_7$	125–139	60–54	6-FAM	KC351507
CM_21R	ACAACACAAGCACACTGCAA					
CM_22F	TGCAGTGTGCTTGTGTTG	$TCT(TC)_4$	134–144	56	VIC	KC351507
CM_22R	TGCCCCTTCTTCTCTCTG					
CM_23F	ACACGGAGCGAGAAAAGTGT	$(AG)_4$	172–192	56	NED	KC351508
CM_23R	GGAAGCTTCAATCCACCAAT					
CM_25F	CAACATTCGGGAGGCTTTTA	$ATGTG(ATG)_2$	300–309	TD 60–54	PET	KC351509
CM_25R	CACCCTCCAAGCACATCAAT					
CM_27F	GTCTCGCGGATCAGTTAAGC	$(CT)_9$	112–120	TD 60–54	6-FAM	KC351510
CM_27R	CATGGCTCGGCTTTACATTT					
CM_31F	TGTGACAAAGTAATGTGAAAGCAA	$(ATG)_3ATATG$	246	56	PET	KC351513
CM_31R	CCCAGCCACTTCAGTTTTCT					

Note: T_a = annealing temperature; TD = touchdown program.

[a] Expected size based on the cloned fragment, excluding the 5′ M13 universal sequence.

TABLE 2. Results of initial microsatellite marker screening in two populations of *Cratylia mollis* and cross-amplification in other species.

	C. mollis										Cross-amplification and transferability	Cross-amplification		
	CMPOP_1 (N = 24)					CMPOP_2 (N = 25)					CMPOP_2 (seeds) (N = 20)	*C. argentea* (N = 9)			
Locus	A	H_o	H_e	PIC	P (HWE)	A	H_o	H_e	PIC	P (HWE)	A	A	*C. bahiensis* (N = 2)	*C. hypargyrea* (N = 2)	*C. intermedia* (N = 2)
CM_1	1	—	—	—	—	3	0.176	0.420	0.377	0.580	2	no	no	no	yes
CM_4	1	—	—	—	—	1	—	—	—	—	2	2	yes	no	yes
CM_5	5	0.458	0.671	0.629	0.317	3	0.042	0.515	0.403	0.919	3	1	yes	no	yes
CM_6	1	—	—	—	—	1	—	—	—	—	1	1	yes	yes	yes
CM_7	1	—	—	—	—	5	0.542	0.603	0.552	0.102	1	2	no	no	no
CM_8	8	0.261	0.526	0.507	0.504	5	0.500	0.548	0.518	0.087	3	3	yes	no	yes
CM_9	1	—	—	—	—	1	—	—	—	—	1	2	yes	no	yes
CM_10	3	0.368	0.481	0.406	0.233	1	—	—	—	—	2	no	no	no	yes
CM_11	9	0.450	0.836	0.817	0.462	11	0.261	0.883	0.870	0.704	10	3	yes	no	yes
CM_15	1	—	—	—	—	5	0.040	0.498	0.462	0.920	2	4	yes	no	yes
CM_18	2	0.545	0.496	0.373	–0.100*	3	0.458	0.478	0.410	0.042	1	2	no	no	yes
CM_19	1	—	—	—	—	1	—	—	—	—	1	3	no	no	yes
CM_20	1	—	—	—	—	3	0.250	0.226	0.212	–0.108*	3	2	yes	yes	yes
CM_21	4	0.792	0.601	0.524	–0.318*	8	0.458	0.666	0.644	0.312	5	2	yes	no	yes
CM_22	1	—	—	—	—	1	—	—	—	—	4	2	yes	no	no
CM_23	1	—	—	—	—	1	—	—	—	—	1	3	yes	no	yes
CM_25	1	—	—	—	—	1	—	—	—	—	3	no	no	no	no
CM_27	n/a	—	—	—	—	n/a	—	—	—	—	5	1	no	no	no
CM_31	1	—	—	—	—	1	—	—	—	—	1	1	yes	no	yes

Note: — = value not calculated; A = number of alleles; H_e = expected heterozygosity; H_o = observed heterozygosity; HWE = Hardy–Weinberg equilibrium; N = number of individuals; n/a = no amplification; PIC = polymorphism information content.

* Significant values ($P < 0.01$).

CONCLUSIONS

This is the first report of microsatellite markers in *C. mollis* and the genus as a whole. The observed polymorphism in 12 of 19 microsatellite loci in *C. mollis*, their successful cross-amplification in *C. intermedia* and *C. bahiensis*, and their transferability to *C. argentea* indicate promising applications for the study of genetic diversity and structure, gene flow, and mating system in the species. These studies can be followed by comparative studies to understand the mechanisms involved in population divergence and speciation in this neotropical genus.

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Appendix 1. Voucher specimens used in this study. All specimens were collected in Brazil and are deposited at the Herbarium of the Universidade Estadual de Feira de Santana (HUEFS). Information shown: taxon, voucher specimen, collection locale/geographic coordinates.

Cratylia mollis Mart. ex Benth.: *J. G. Rando 1257*. Individual cultivated at the Universidade Estadual de Feira de Santana (Feira de Santana, Bahia).

Cratylia mollis Mart. ex Benth. (Population 1): *G. Pereira-Silva 8450* (Barra, Bahia: 10°47′27″S, 042°50′05″W).

Cratylia mollis Mart. ex Benth. (Population 2): *G. Pereira-Silva 9165* (Casa Nova, Bahia: 09°24′55″S, 41°08′22″W).

Cratylia argentea (Desv.) Kuntze: *C. Snak 878* (Rio Verde, Mato Grosso do Sul: 18°58′14.8″S, 54°49′17.6″W), *902* (Pedro Gomes, Mato Grosso do Sul: 18°10′53.2″S, 54°38′30.6″W), *965* (Loreto, Maranhão: 07°19′07.1″S, 45°08′56.1″W), *989* (Carolina, Maranhão: 07°13′18.9″S, 47°25′50.7″W), *L. C. P. Lima 614* (Cáceres, Mato Grosso), *L. P. Queiroz 13975* (Posse, Goiás: 14°17′32.999″S, 46°24′14.000″W), *10570* (Barra do Bugres, Mato Grosso: 15°11′27.001″S, 57°6′28.001″W), *10281* (Posse, Goiás: 14°17′43.000″S, 46°24′19.001″W), *10405* (Barra do Garças, Mato Grosso: 15°49′44.002″S, 52°29′59.999″W).

Cratylia bahiensis L. P. Queiroz: *G. Pereira-Silva 9081* (Caetité, Bahia: 14°07′51″S, 42°23′35″W), *9129* (Abaíra, Bahia: 13°16′06″S, 41°41′27″W).

Cratylia intermedia (Hassl.) L. P. Queiroz: *C. Snak 592* (Diamante do Oeste, Paraná), *1052* (Formosa do Oeste, Paraná: 24°12′52.86″S, 53°19′39.14″W).

Cratylia hypargyrea Mart. ex Benth.: *J. Carvalho-Sobrinho 2948* (Olivença, Bahia: 15°6′56.002″S, 39°1′12.001″W), *L. P. Queiroz 2847* (Ilhéus, Bahia: 14°56′00.001″S, 39°2′00.001″W).