Microsatellite Markers Isolated from Cabomba aquatica s.l. (Cabombaceae) from an Enriched Genomic Library

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Source: Applications in Plant Sciences, 3(11)

Published By: Botanical Society of America

URL: https://doi.org/10.3732/apps.1500076

Applications in Plant Sciences

Applications in Plant Sciences 2015 3(11): 1500076

Primer Note

Microsatellite markers isolated from *Cabomba aquatica* s.l. (Cabombaceae) from an enriched genomic library[1]

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- *Premise of the study:* Microsatellite primers were designed for the submersed aquatic plant *Cabomba aquatica* s.l. (Cabombaceae) and characterized to estimate genetic diversity parameters.
- *Methods and Results:* Using a selective hybridization method, we designed and tested 30 simple sequence repeat loci using two natural populations of *C. aquatica* s.l., resulting in 13 amplifiable loci. Twelve loci were polymorphic, and alleles per locus ranged from two to four across the 49 *C. aquatica* s.l. individuals. Observed heterozygosity, expected heterozygosity, and fixation index varied from 0.0 to 1.0, 0.0 to 0.5, and −1.0 to −0.0667, respectively, for the Manaus population and from 0.0 to 1.0, 0.0 to 0.6, and −1.0 to 0.4643 for the Viruá population.
- *Conclusions:* The developed markers will be used in further taxonomic and population studies within *Cabomba*. This set of microsatellite primers represents the first report on rapid molecular markers in the genus.

Key words: Amazon; *Cabomba aquatica* s.l.; Cabombaceae; molecular markers; simple sequence repeat (SSR).

Cabomba Aubl. is a small genus in the family Cabombaceae comprising strictly aquatic plants restricted to Neotropic and adjoining warmer temperate zones (Ørgaard, 1991). The genus contains six (Fassett, 1953) or five (Ørgaard, 1991) species. The last and more complete taxonomic study was made by Ørgaard in 1991, in which five species were recognized, as she synonymizes *C. schwartzii* Rataj under *C. aquatica* Aublet. *Cabomba aquatica* s.l. is distributed along northern, northeastern, and southeastern regions of Brazil and northern South American countries, occurring in habitats such as floodplains, floodplain lakes, creeks, ponds, and swampy areas where sufficient light is available. The color of the flower, morphology of the floating leaves, phyllotaxy of the submerged leaves, and seed size and shape are the main characters used for recognizing species (Ørgaard, 1991). Despite the great contribution of the study by Ørgaard (1991), identification of *Cabomba* species is still problematic due to vegetative similarity among the taxa, the presence of few morphological characters that are useful in delimiting species, and the necessity of both flower and seed to differentiate some species. Grown for its ornamental value, *Cabomba* species were one of the most important plants to be commercialized for a long period in the history of aquarism (Francisco and Barreto, 2007). However, the rapid development of *Cabomba* plants in water reservoirs can negatively affect the water flow in hydroelectric turbines and irrigation channels and can reduce the navigability of watercourses. Every year, countries like Australia and the United States spend millions of dollars on its control to minimize the damage (Francisco and Barreto, 2007). On the other hand, species of *Cabomba* are important elements of water plant vegetation with a high primary production rate. The plants are ecologically important as a food source and as hiding places for several vertebrate and invertebrate species. They also produce considerable biomass and act as a nutrient reservoir (Esteves, 1998; Silva and Leite, 2011). For these reasons, a set of rapid molecular markers is needed in *C. aquatica* s.l. In this study, we report the development of 13 microsatellite loci for the species to subsidize further taxonomic and population studies within *Cabomba.*

METHODS AND RESULTS

The total genomic DNA was extracted from floating leaf tissue dried in silica gel using a cetyltrimethylammonium bromide (CTAB) method, based on Doyle and Doyle (1987), and then digested with *Afa*I restriction enzyme. Microsatellite DNA loci were isolated from one individual from Parque Nacional do Viruá, Roraima, Brazil (*Barbosa, T. D. M. 1230 & Costa, S. M.* [UEC 154811]) (Appendix 1), as described in Billotte et al. (1999). Enrichment was performed using a hybridization-based capture with $(CT)_8$ and $(GT)_8$ biotin-linked probes and streptavidin-coated magnetic beads (MagneSphere Magnetic Separation Products; Promega Corporation, Madison, Wisconsin, USA). The enriched fragments were amplified by PCR, and the amplification products were cloned into pGEM-T Easy Vector (Promega Corporation). Competent XL1-Blue *Escherichia coli* (Stratagene, Agilent Technologies, Santa Clara,

[1]Manuscript received 30 June 2015; revision accepted 24 July 2015.

The authors thank S. M. Costa for help during fieldwork, the staff of the Parque Nacional do Viruá and Instituto Nacional de Pesquisas da Amazônia (INPA) for field support, and A. P. Souza for technical support for the laboratory infrastructure. T.D.M.B. is grateful to the Post-Graduate program in Plant Biology of the Instituto de Biologia (UNICAMP) and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) for the scholarship; M.C.E.A. is a Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) research fellow. This work was financed by CAPES (PNADB 457/2010).

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doi:10.3732/apps.1500076

TABLE 1. Characteristics of 13 successfully amplified SSR loci developed for *Cabomba aquatica* s.l.

Locus	Primer sequences (5′–3′)	Repeat motif	Allele size range (bp)	T_a (°C)	GenBank accession no.
CS01	F: CGAACCTTTTGTTTCTCCTT R: CTCCTGAAGTTTTCCCATTT	$(CT)_{18}$	170–191	53.6	KT026291
CS02	F: TGCAAACTCTTCCCTGTCTA R: AGCGTAATGGGTAACTACG	$(CT)_6(CA)_{11}T(AC)_7$	178–196	57.1	KT026292
CS03	F: AACTGTTTGGAAATCCGACT R: CCCTCCCATACCATCAATA	$(AC)_7$	146–152	55.2	KT026293
CS04	F: ACACCCTCCAAGATGAATTT R: TAGCCAGACACTCCGAAATA	$(AC)_8$	224–240	53.6	KT026294
CS05	F: GATTGGTCTCAGGTGTTGAA R: TCTCTTGGTGTCAAAGCACT	$(GA)_5$	198–202	53.6	KT026295
CS06	F: ACATAGGAACGTCCTTCTCC R: GTAGGCTTTGACGACCTTCT	$(AC)_8(GA)_9$	240–250	53.6	KT026296
CS07	F: CATCCTTAGAACACCCGACT R: GCAGGGCATTGATTAGTTTT	$(AC)_{10}$	220–226	55.2	KT026297
CS08	F: GACACTCTTAACACTCCTCCTG R: ACGTTATCAAAGTCGCTTCC	$(GT)_7$	150–152	52.0	KT026298
CS09	F: CAAGTCAAGCTGTGTTTGGT R: GCACTCAACCATCATCAACT	$(TG)_{10}(GA)_{11}$	190–206	60.0	KT026299
CS10	F: GCCACAAGAAGATCCATACA R: GAAAGTGCTTCTGTCCATCA	$(AC)_8$	202–204	58.7	KT026300
CS11*	F: GAGGTGCATCTTTTCTTCCT R: CTCAATAAAGCGGGGAAC	$(CA)_5$	228–230	55.2	KT026301
CS12	F: ACCTTGTCGTCTGTAAGCTG R: TTGCAGGTCATATCCTTGTT	$(AC)_8$	220–228	55.2	KT026302
CS13	F: GAAGGTTCTGAAACCCTGAT R: CATTTGGCACGAGCTAATAC	$(TC)_{21}$	184–252	60.0	KT026303

Note: T_a = specific annealing temperature.
* Monomorphic locus.

California, USA) were transformed with the recombinant plasmids and cultivated on agar medium containing ampicillin and 100 µg/mL of X-galactosidase. Ninety-six recombinant colonies were selected using blue/white screening and sequenced in an automated ABI 3500xL Genetic Analyzer (Perkin Elmer–Applied Biosystems, Foster City, California, USA) using T7 and SP6 primers and the BigDye Terminator version 3.1 Cycle Sequencing Kit (Perkin Elmer–Applied Biosystems). Approximately 86 sequenced clones presented microsatellite motifs, from which 30 primer pairs were designed, using Primer3Plus (Untergasser et al., 2007). As a criterion for the selection of simple sequence repeats (SSRs), sequences that showed at least five dinucleotide repeats, four trinucleotide repeats, or three tetra-, penta-, and hexanucleotide repeats were selected, giving preference to motifs with more repetitions. From the 30 primer pairs developed, 13 did not successfully amplify in PCR and four did not show conclusive results. Thirteen primer pairs amplified PCR products, from which one pair was monomorphic (CS11). The characteristics of the primer pairs and the optimal annealing temperature are given in Table 1. These revealed a two-banded pattern, which is typical for diploid organisms. Preliminary cytogenetic studies reported $2n = 26$, 52, or 104 chromosomes for *C. aquatica* s.l. (Ørgaard, 1991), being diploid, tetraploid, and octoploid, respectively.

Polymorphism was tested with total DNA of 49 individuals from two populations: (1) a lake on the campus of the Instituto Federal do Amazonas (IFAM), municipality of Manaus, Amazonas State, Brazil (3°06′07″S, 60°01′30″W; $n = 29$), and (2) Parque Nacional do Viruá, municipality of Caracaraí, Roraima State, Brazil (1°24′44″N, 60°13′00″W; $n = 20$). The microsatellite fragments were amplified by PCR containing 20 ng of template DNA, 2.5 µL of 1× PCR buffer (20 mM Tris HCl [pH 8.4] and 50 mM KCl), 2.0 µL (10.0 mM) of forward and reverse primer, 1.5 µL of dNTP mix (2.5 mM of each base), 1.5 µL of $MgCl_2$ (1.5 mM), and 1 unit of *Taq* DNA polymerase (Fermentas, Thermo Fisher Scientific, Pittsburgh, Pennsylvania, USA). The PCR program for all loci amplification consisted of an initial denaturation at 95°C for 3 min, followed by 39 cycles of denaturation at 95°C for 40 s, annealing at specific temperature for 40 s (Table 1), extension at 72°C for 50 s, and a final extension at 72°C for 8 min. PCR products were separated by electrophoresis in denaturing acrylamide gels and silver stained (Creste et al., 2001). The molecular size of the fragments was estimated using a 10-bp ladder (Invitrogen, Carlsbad, California, USA).

For each population, we calculated the number of alleles per locus (A), expected heterozygosity (H_e), observed heterozygosity (H_o), and fixation index (F) using the R package HIERFSTAT (Goudet, 2005). Adherence to Hardy–Weinberg equilibrium (HWE) and linkage disequilibrium (LD) among loci was tested using the Markov chain method in the software GENEPOP version 4.2 (Raymond and Rousset, 1995). Deviations from HWE in the two populations could derive from asexual reproduction, which is reported to be high for *Cabomba* (Ørgaard, 1991), and from environmental anthropization. No LD was detected between pairs of loci after Bonferroni correction for multiple tests. The number of alleles per locus in the 12 polymorphic loci ranged from two to four across the 49 *C. aquatica* s.l. individuals. H_o, H_e, and F vary from 0.0 to 1.0, 0.0 to 0.5, and −1.0 to −0.0667, respectively, in the Manaus population, and from 0.0 to 1.0, 0.0 to 0.6, and −1.0 to 0.4643 in the Viruá population (Table 2).

TABLE 2. Genetic diversity values for 49 individuals of *Cabomba aquatica* s.l. across 12 polymorphic SSR loci.

	Manaus ($n = 29$)				Viruá ($n = 20$)			
Locus	A	H_o	H_e	F	A	H_o	H_e	F
CS01	1	0.00	0.00	NA	3	0.45	0.60	0.2361
CS02	2	1.00	0.50	−1.0000*	1	0.00	0.00	NA
CS03	2	0.69	0.45	−0.5294	2	1.00	0.50	−1.0000*
CS04	2	1.00	0.50	−1.0000*	2	1.00	0.50	−1.0000*
CS05	2	0.12	0.12	−0.0667	2	0.20	0.18	−0.1111
CS06	2	1.00	0.50	−1.0000*	1	0.00	0.00	NA
CS07	1	0.00	0.00	NA	1	0.00	0.00	NA
CS08	2	0.39	0.32	−0.2444	2	0.17	0.15	−0.0909
CS09	2	1.00	0.50	−1.0000*	2	0.27	0.50	0.4643
CS10	1	0.00	0.00	NA	1	0.00	0.00	NA
CS12	1	0.00	0.00	NA	2	1.00	0.50	−1.0000*
CS13	2	0.76	0.50	−0.5298	2	1.00	0.50	−1.0000*

Note: A = number of alleles per locus; F = fixation index; H_e = expected heterozygosity; H_o = observed heterozygosity; n = sample size for each population; NA = not applicable (i.e., monomorphic locus).
* Departs significantly from Hardy–Weinberg equilibrium after Bonferroni correction ($\alpha = 0.0041$).

CONCLUSIONS

These are the first SSR markers developed for the *Cabomba* genus. These loci will allow us to investigate the genetic structure of *C. aquatica* s.l. populations alongside morpho-anatomical studies to reconsider whether *C. schwartzii* should be recognized as a distinct species. They will also provide support for the adequate management of this ecologically important species and may be instrumental for further ecological research.

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Appendix 1. Voucher and location information for *Cabomba aquatica* s.l. populations used in this study. One voucher was collected for each population used; all vouchers were deposited in the herbarium of the Universidade Estadual de Campinas (UEC), Campinas, São Paulo, Brazil.

Voucher no.	Collection date	Locality	Geographic coordinates	Herbarium ID
Barbosa, T. D. M. 1230 & Costa, S. M.	20 July 2010	Parque Nacional do Viruá, Caracaraí, Roraima	1°24′44″N, 60°13′00″W	UEC 154811
Barbosa, T. D. M. 1479	30 March 2011	IFAM lake, Manaus, Amazonas	3°06′07″S, 60°01′30″W	UEC 185233