PRIMER NOTE

MICROSATELLITE MARKERS ISOLATED FROM *CABOMBA AQUATICA* S.L. (CABOMBACEAE) FROM AN ENRICHED GENOMIC LIBRARY

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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* Ratat 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 *AflII* 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, CA) were transformed with the plasmid DNA. Plasmids were isolated from overnight cultures using a Wizard Plus SV Miniprep DNA Purification System (Promega Corporation). Four microsatellite loci were isolated from each of four individuals of *C. schwartzii* and *C. aquatica* s.l. (Manaus) sampled in February and March 2011, respectively.

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doi:10.3732/apps.1500076

Applications in Plant Sciences 2015 3(11): 1500076; http://www.bioone.org/loi/apps © 2015 Barbosa et al. Published by the Botanical Society of America.

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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 clones 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 were selected, giving preference to motifs with more repetitions.

From the 30 primer pairs developed, 13 did not successfully amplify in PCR repeats, four trinucleotide repeats, or three tetra-, penta-, and hexanucleotide 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. individuals. From two to four across the 49 C. aquatica s.l. individuals, H\(_e\), \(H_o\), 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 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: CGAACCTTGTGTTTCTCCTT | (CT)\(_{18}\) | 170–191 | 53.6 | KT026291 |
| R: ACCTCTAAATTTTTATGATG | (AC)\(_2\) | 178–196 | 57.1 | KT026292 |
| CS02  | F: GACAACTCTTCTCTCTGTA | (CT)\(_{10}\)(CA)\(_7\)(AC)\(_7\) | 146–152 | 55.2 | KT026293 |
| R: AGGTAATGGTAACTGTTAC | (AC)\(_6\) | 224–240 | 53.6 | KT026294 |
| CS03  | F: AACTGGTGGAAATCCGACT | (AC)\(_5\) | 198–202 | 53.6 | KT026295 |
| R: CCCCCTCTACGCAATGAT | (GA)\(_3\) | 240–250 | 53.6 | KT026296 |
| CS04  | F: ACCATGAGCTCTCCCTTC | (AC)\(_3\)(GA)\(_3\) | 220–226 | 55.2 | KT026297 |
| R: GCAGGGCCTTAGATTGTAG | (AC)\(_{10}\) | 190–206 | 52.0 | KT026298 |
| CS05  | F: GACACTTCTTTACTCTCTTG | (GT)\(_2\) | 202–204 | 58.7 | KT026300 |
| R: ACCTAGTAAAGTCGTCCCT | (TG)\(_{10}\)(GA)\(_{11}\) | 228–230 | 55.2 | KT026301 |
| CS06  | F: GCACCAAGAAGATCTACATA | (AC)\(_{8}\) | 190–206 | 57.1 | KT026302 |
| R: GCAGCTCAGACTCATACACT | (GA)\(_3\) | 220–228 | 55.2 | KT026303 |
| CS07  | F: GCACACCAAGAAGATCTACATA | (AC)\(_{8}\) | 202–204 | 58.7 | KT026300 |
| R: GAAAGTCTCTTCCTGCATAC | (GA)\(_3\) | 228–230 | 55.2 | KT026301 |
| CS11* | F: GAGTGTACCTTTTCCTTCTCTT | (AC)\(_{5}\) | 202–206 | 58.7 | KT026300 |
| R: CTCAATATAGCGGGGAGAAC | (GC)\(_{13}\) | 220–228 | 55.2 | KT026301 |
| CS12  | F: AACTGGTGTCGTTGATTAGGG | (AC)\(_{8}\)(GA)\(_{9}\) | 190–206 | 52.0 | KT026298 |
| R: TTGAGGCTGATATCCCTTGGTT | (TG)\(_{10}\)(GA)\(_{11}\) | 220–228 | 55.2 | KT026302 |
| CS13  | F: GAGGTTCTGGAAAGAATCTGAT | (TC)\(_{21}\) | 184–252 | 60.0 | KT026303 |
| R: CATTTTGCAAGCACTGTA | (TC)\(_{21}\) | 202–206 | 58.7 | KT026300 |

**Note:** \(T_a\) = specific annealing temperature.

*Monomorphic locus.

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

| Locus | Manaus (n = 29) | Viruá (n = 20) |
|-------|----------------|----------------|
| \(A\) | \(H_e\) | \(H_o\) | \(F\) | \(A\) | \(H_e\) | \(H_o\) | \(F\) |
| 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  | 2 | 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 |