ONLINE RESOURCES

Development, characterization and cross-amplification of microsatellite markers for *Chrysolaena obovata*, an important Asteraceae from Brazilian Cerrado

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Introduction

Chrysolaena obovata (Less.) Dematt. [=*Vernonia herbacea* (Vell.) Rusby] is an Asteraceae species widely spread in the Brazilian Cerrado. The underground organs of this perennial herb, known as rhizophores, store up to 80% of its dry mass as inulin-type fructans (Carvalho and Dietrich 1993). Fructans are well known as storage carbohydrates able to confer tolerance to drought and low temperatures (Carvalho *et al.* 2007). Inulin is also recognized as a functional food ingredient, due to its beneficial roles in human health (Ritsema and Smeekens 2003).

The Cerrado biome occupies 21% of the Brazilian territory, hosts a high biodiversity and has been classified as one of the world's hotspots (Simon *et al.* 2009). Because of its intensive exploitation, the Cerrado area was drastically reduced leading to intense fragmentation of this biome (Sano *et al.* 2007). This fragmentation may result in loss of genetic variability due to genetic drift, inbreeding and reduced gene flow (Zucchi *et al.* 2003) . Thus, the characterization of genetic

structure and population dynamics of Cerrado species is crucial for the development of management strategies and conservation programs.

Asteraceae is the largest family among the angiosperms (Bremer 1994) with great importance and predominance in the herbaceous layer of the Cerrado (Batalha *et al.* 2001). Despite the ecological and economical importance of this family, little genetic information is available for its natural populations. Additionally, in recent years, efforts to develop molecular markers have mainly focused on economically important Asteraceae species like sunflower (Chapman *et al.* 2007), lettuce (Van de Wiel *et al.* 1999), and safflower (Chapman *et al.* 2009). Although there have been increasing efforts to characterize the genetic diversity in the Cerrado vegetation, most studies refer to tree or shrub species. More recently, SSR markers were developed for *Lychnophora ericoides* Mart. (Rabelo *et al.* 2011) and *L. pinaster* Mart. (Haber *et al.* 2009), two endemic and endangered Brazilian Asteraceae species.

Nevertheless, the overall knowledge on the levels of genetic diversity and structure of natural Cerrado populations is insufficient for conservation strategies requirement. The aim of this study was to develop SSR markers to estimate genetic diversity and structure in natural populations of *Chrysolaena obovata* and to evaluate cross-species amplification of these markers in 17 Asteraceae species from seven different tribes.

Material and methods

Total genomic DNA (table 1) was extracted from silica gel-dried leaf samples of *C. obovata* and other 17 species of Asteraceae, according to the procedure of Allen *et al.* (2006), with minor modifications. Briefly, the extraction buffer was modified by the addition of 2% β-mercaptoethanol (v/v) and 2% polyvinylpyrrolidone (PVP, w/v, M_r10,000). Also, an additional cleaning step in equal volume of chloroform: isoamyl alcohol (24:1) was included before precipitation in isopropyl alcohol. DNA concentration was measured on a nanophotometer (Implen) and quality was checked on TAE 1X agarose gels stained with Gelred® (Biotium). DNA of one specimen of *C. obovata* sampled in a Cerrado area in São Paulo state (22º18'S/47º11'W) was used to construct a microsatellite-enriched library according to the protocol adapted from Billotte *et al.* (1999). Approximately 5µg of DNA was digested using the restriction enzyme *RsaI* (Promega), and the fragments were ligated to the adaptors *Afa21* (5'-CTCTTGCTTACGCGTGGACTA-3') and *Afa25* (5'-TAGTCCACGCGTAAGCAAGAGCACA-3'). Resulting fragments were PCR-amplified using Rsa21-specific primers. The library was then enriched for $(CT)_8$ and $(GT)_8$ repeats, using 5'-biotinylated microsatellite probes. Microsatellite-enriched fragments were captured by streptavidin magnetic beads (Promega), cloned into pGEM-T Easy Vector (Promega), and subsequently

transformed into competent XL1-Blue *Escherichia coli* cells. Positive clones (60) were selected for sequencing reactions containing 200 ng of plasmid DNA, 0.5 pmol primer (SP6 or T7), 0.4 μL of BigDye Terminator mix (version 3.1; Applied Biosystems), 1 mM $MgCl_2$, and 40 mM Tris-HCl (pH 9.0). The sequencing reactions were performed in a thermal cycler (MJ Research, BioRad) under the following conditions: 1 min at 95 °C, followed by 26 cycles of 20 s at 95 °C, 20 s at 50 °C, and 4 min at 60 °C. Sequencing was performed in an ABI 3500xL automated sequencer (Applied Biosystems). The resulting sequences were screened for SSR repeats using the Gramene Project's SSR identification tool (www.gramene.org/db/markers/ssrtool) and consensus sequences were generated using the CAP3 (Huang and Madan 1999). Vector sequences were discarded using the BLAST VecScreen tool (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and primers were designed with Primer3 (http://frodo.wi.mit.edu/primer3/) (Rozen and Skaletsky 2000). Primer sequences and the corresponding GenBank accession numbers are shown in table 2.

The primer pairs with consistent amplification patterns were used for genotyping 87 *C. obovata* individuals from three natural populations (table 1). For genotyping, two amplification reactions were carried out for each locus in a Mastercycler thermocycler (Eppendorf). The first-round PCR was carried out in a volume of 25 μL containing 25 ng DNA, 1X GoTaq®Flexi Buffer (Promega), 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 0.24 mM of each primer, and 1 U of Taq polymerase (Promega). An initial denaturation at 95 °C for 3 min was followed by 35 cycles of denaturation at 95 °C for 45 s, annealing at an optimal temperature (table 2) for 45 s, extension at 72 °C for 45 s, and a final extension at 72 °C for 5 min. Subsequently, a second PCR reaction was performed according to the method developed by Schuelke (2000), with a sequence-specific forward primer with M13(-21) tail (5'-CACGACGTTGTAAAACGACA-3') at its 5′ end. The second PCR was carried out in a volume of 12.5 μL containing 0.5 μL of the first PCR product, 1X GoTaq®Flexi Buffer (Promega), 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 0.04 mM of forward primer, 0.16 mM of reverse primer, 0.16 mM of fluorescent-labeled (IR700 or IR800) M13 primer, and 1 U of Taq polymerase (Promega). PCR amplification was performed as follows: initial denaturation at 94°C for 5 min, followed by 30 cycles at 94 °C for 30 s, annealing at 50 to 60 °C for 45 s (according to the primer temperature, table 2), 72 °C at 45 s, followed by 8 cycles at 94 °C (30 s), 53 °C (45 s) and 72 °C (45 s), and a final extension at 72 °C for 10 min. A non-template negative control was included in each PCR amplification. The PCR products were analyzed by 1.5 % agarose gel electrophoresis stained with Gelred® (Biotium), to confirm product amplification and integrity, as a preliminary selection for further genotyping. Polymorphisms were detected in 5% (v/v) polyacrylamide gels containing 8 M of urea in a semi-automated sequencer (LI-COR 4300S DNA Analysis System; LI-COR Biosciences). The loci were genotyped using SagaGT software (LI-COR Biosciences), and allele sizing was performed by comparing alleles with IRDye-700 and

IRDye-800 size standards (50 - 350 bp, LI-COR Biosciences) (figure 1).

For characterization of the polymorphic loci, genetic parameters such as allele number (*A*), observed heterozygosity (H_O), expected heterozygosity (H_E), and polymorphism information content (*PIC*) were calculated for each locus using Cervus 3.0 (Kalinowski 2005). Deviations from Hardy-Weinberg equilibrium (HWE) were evaluated using the exact probability tests of the GENEPOP software on the web (version 1.2) (http://wbiomed.curtin.edu.au/genepop/index.html) (Raymond and Rousset 1995). Linkage disequilibrium was tested in FSTAT 2.9.3.2 (Goudet 2001). The statistical significance was adjusted for multiple testing using a sequential Bonferroni correction for multiple comparisons at 5% significance. The presence of null alleles was determined using Micro-Checker 2.2.3 (Van Oosterhout *et al.* 2004). To analyze the cross-amplification of the nine polymorphic SSRs among other 17 Asteraceae species, PCR amplification was performed as described for *C. obovata*. The loci were considered successfully amplified when at least one band of the expected size was visualized in at least one of the individuals from the Asteraceae species tested.

Results and discussion

From the 45 *C. obovata* microssatelite markers tested, 33 were successfully amplified by PCR. The nine polymorphic loci were further used in the analyses of genetic diversity of 87 individuals from three populations of *C. obovata*. The number of alleles ranged from three to eight (table 2), with an average of 5.11 alleles per locus. This average number of alleles is consistent with that observed for Eudicots, and more specifically, to the Asterid clade, as recently demonstrated by a meta-analysis of 6782 plant genomic microsatellite markers (Merritt *et al.* 2015). The nine polymorphic SSRs showed a moderate mean value of *PIC* (0.4344), ranging from 0.087 to 0.694. The observed heterozygosity ranged from 0.093 to 0.909 and the expected heterozygosity ranged from 0.090 to 0.732 per locus (table 2). No linkage disequilibrium was detected between pairs of loci after Bonferroni correction for multiple tests ($P = 0.0006$). Four loci (C12, D8, F3-SSR2 and VH10) showed significant deviation from the HWE and evidence of null allele was found at the loci VH10 in PESP population (table 2).

Cross-amplification analysis revealed that all the polymorphic loci successfully amplified in at least five of the 17 Asteraceae species (table 3). The transferability rate of the markers ranged from 29.4% to 94.1% (table 3), indicating their potential application in genetic studies of other Asteraceae. Previous studies reported reasonably high levels of transferability across taxa, showing that the success rate decreases as the genetic distance increases (Barbará *et al.* 2007, Chapman *et al.* 2009). Interestingly, the cross-amplification efficiency did not decrease with the increase in evolutionary distances since tribes rather distant from Vernonieae, such as Neurolaeneae and

Heliantheae, showed higher percentages of cross-amplification (88.9%) than Gnaphalieae (77.8%), which is closely related to Vernonieae (figure 2). Nevertheless, the rate of primer cross-amplification does not depend only on the phylogenetic distance among species, but also on genome size and complexity and on the localization in the coding, intronic or intergenic DNA (Barbará *et al.* 2007).

These are the first microsatellite markers developed for *C. obovata*, a non-model Asteraceae species with a high potential for the industrial production of inulin. The overall results suggest that regions flanking the repeats are highly conserved and that SSR markers described here are suitable for phylogenetic investigations within the Asteraceae family. This is a highly important result, considering the high costs and time required for the development of species-specific primers and the scarce information about microsatellite markers for native and endemic species (Barbará *et al.* 2007).

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Table 1. Sampling location of the three populations of *Chrysolaena obovata* and of the other 17 Asteraceae species.

Subfamily/Tribe *Species*	Location	N	Geographic coordinate
Asteroideae/Coreopsideae			
Bidens gardneri Baker	EEcSB/SP	3	22°47.231'S / 49°14.316'W
Asteroideae/Eupatorieae			
Chromolaena latisquamulosa (Hieron.) R.M.King & H.Rob	EEcSB/SP	3	22°47.231'S / 49°14.316'W
Asteroideae/Gnaphalieae			
Achyrocline sp.	EEcSB/SP	3	22°47.231'S / 49°14.316'W
Achyrocline satureioides (Lam.) D.C.	PESP/GO	3	15°47.449'S / 48°50.191'W
Asteroideae/Heliantheae			
Aspilia foliacea (Spreng.) Baker	EEP/MG	3	19°10.928'S / 48°23.576'W
Dimerostemma vestitum (Baker) S.F.Blake	PESD/GO	3	16°04.169'S / 50°10.860'W
Helianthus tuberosus L.	PEFI/SP	3	23°38’08”S / 46°38´00”W
Viguiera discolor Baker	EEP/MG	3	19°10.922'S / 48°23.598'W
Ichthyothere mollis Baker	PESP/GO	3	15°47.449'S / 48°50.191'W
Ichthyothere terminalis (Spreng.) S.F.Blake	PESP/GO	3	15°47.449'S / 48°50.190'W
Asteroideae/Neurolaeneae			
Calea platylepis Sch.Bip. ex Baker	EEP/MG	3	19°10.207'S / 48°23.430'W
Calea quadrifolia Pruski & Urbatsch	PESD/GO	3	16°04.157'S / 50°10.863'W
Asteroideae/Polymnieae			
Smallanthus sonchifolius (Poepp) H. Robinson	PEFI/SP	3	23°38’08”S / 46°38´00”W
Cichorioideae/Vernonieae			
Chrysolaena simplex (Less.) Dematt.	PESP/GO	3	15°47.443'S / 48°50.186'W
Chrysolaena obovata (Less.) M. Dematt.	SI/MG	30	16°57.423'S / 43°24.051'W
	PESP/GO	27	15°47.250'S / 48°50.090'W
	EEP/MG	30	19°10.921'S / 48°23.626'W
Lessingianthus buddleiifolius (Mart. ex DC.) H.Rob.	PESD/GO	3	16°04.177'S / 50°10.824'W

Vernonia fagifolia Gardner	EEP/MG	2	19°10.216'S / 48°23.454'W
Vernonia ferruginea (Less)	EEP/MG	1	19°10.901'S / 48°23.661'W

Estação Ecológica de Santa Bárbara – São Paulo (EEcSB/SP); Parque Estadual da Serra dos Pireneus – Goiás (PESP/GO); Estação Ecológica do Panga – Minas Gerais (EEP/MG); Parque Estadual da Serra Dourada – Goiás (PESD/GO); Parque Estadual das Fontes do Ipiranga - São Paulo (PEFI/SP); Serra de Itacambira, Minas Gerais (SI/MG)

Table 2. Characteristics of nine polymorphic microsatellite loci genotyped in 87 individuals from three populations of *Chrysolaena obovata*.

Locus	Primer sequence (5'- 3')	Repeat motif	T_a	Size (bp)	*A*	H_O	H_E	*PIC*	Accession number
C3	F:TTCGACACACCCCTAAAACC R: GCTAAATTCGCCTCCTTTTG	$(AT)_4$	60	287 - 309	6	0.353	0.312	0.295	KR920052
C10	F:TCCACATACCGGATCTAGGC R:CCTATGCATGCACTTGGTGT	$(AG)_4T(TG)_4G(TG)$	55	243 - 275	5	0.247	0.224	0.208	KR920057
C12	F:CACGGTTTAATCACGGCTTG R: CTATGCTGCCCATCCCTTT	$(AG)_3AT(GA)_4N_4(AG)_4N_3(GA)_4$	50	229 - 241	7	0.821	0.709	0.663**	KR920059
D8	F:CCACGTGTAGGGTTAAAATG R:GAATTGGATCATGATGCTTG	$(TG)_5N_{35}(CA)_{14}$	50	132 - 136	3	0.828	0.557	0.481**	KR920067
E5	F: GCACGAACTAGTATCCGACA R: GGCTAAGTCAAATATGTGTGC	$(CA)_2A(CA)_8$	55	205 - 225	4	0.671	0.628	0.552	KR920073
F3-SSR2	F: AGGTTTAAGAAGGGGTTGAG R: TCAATCTCCTTCACTCACTG	$(TG)_{11}N_{16}(GT)_3N_3(TG)_4\ N_{40}\ (TG)_4$	50	280 - 282	8	0.909	0.732	0.694**	KR920079
F4	F: TAGGGGTGGGTGGATTTAG R: ATCGTGCAGGATATCTAGGG	$(TC)_3$	55	144 - 160	3	0.422	0.377	0.309	KR920080
F5	F: CGACAGTTAGGCTCCAAAGA R: CCTCCCATACACCAATAACG	$(TG)_4$	55	302 - 348	3	0.093	0.090	0.087	KR920081
VH10	F: TCTTGGTCTGAGAGAGGTGT R: AACGAGTGTGATCAGCAGAG	$(CA)_7$	60	274 - 296	7	0.701	0.665	0.621*	KR920090

T_a, annealing temperature (°C); *A*, number of alleles per locus; H_O, observed heterozygosity; H_E, expected heterozygosity; *PIC*, polymorphic information content;

** significant deviation from Hardy-Weinberg equilibrium HWE with $P < 0.001$ and * with $P < 0.01$.

Table 3. Cross-amplification of nine microsatellite loci of *Chrysolaena obovata* in 17 Asteraceae species from seven different tribes.

Tribe/Species	N	C10	E5	D8	F4	C12	F5	F3-2	C3	Vh10	No. of loci
Vernonieae											
C.simplex	3	+++	++	++	++	+++	++	++	++	-	8
L.buddleiifolius	3	++	++	+++	+++	+++	+++	+++	+++	+	9
V. fagifolia	2	++	++	++	++	++	-	-	-	-	5
V. ferruginea	1	+	-	+	+	-	-	+	-	+	5
Gnaphalieae											
Achyrocline sp.	3	+	-	+++	+	+	-	++	-	-	5
A. satureioides	3	+++	+++	++	++	++	++	++	-	-	7
Coreopsideae											
B. gardineri	3	++	+	++	+	+++	+	-	-	-	6
Neurolaeneae											
C. platylepis	3	+++	+++	+++	+++	+++	-	++	-	++	7
C. quadrifolia	3	+++	++	+++	+++	+++	+++	+++	-	++	8
Polymnieae											
S. sonchifolius	3	-	-	-	-	-	-	-	-	+	1
Heliantheae											
A. foliacea	3	++	++	+	++	++	-	-	-	+	6
D. vestitum	3	+	-	-	-	+++	-	-	+++	-	3
H. tuberosus	3	+++	+++	-	+++	+++	++	-	+++	+++	7
V. discolor	3	++	+	+	+	+	-	-	-	+	6
I. mollis	3	++	+++	++	+	+	+	-	-	-	6
I. terminalis	3	+	+	++	+	++	+	+	+	-	8
Eupatorieae											
C. latisquamulosa	3	++	-	+	-	++	+	+	-	-	5
% of cross transferability		**94.1**	**70.6**	**82.3**	**82.3**	**88.2**	**52.9**	**52.9**	**29.4**	**47.1**	

NOTE — Species codes are given in Table 1. Symbols '+++','++','+' indicate successful amplification in three, two and one indivuals respectively and '-' indicates no amplification.

Figure caption

Figure 1. Representative gel image of the pattern obtained for locus F5 genotyping. Eighty-seven accessions from three *C. obovata* populations were analyzed on a 5% polyacrylamide gel with 8 M of urea in a LI-COR semi-automated sequencer. Lane M: Molecular Marker IRDye-800 Size Standard. The amplified SSR DNA bands representing different alleles were scored using SagaGT software (LI-COR Biocsciences).

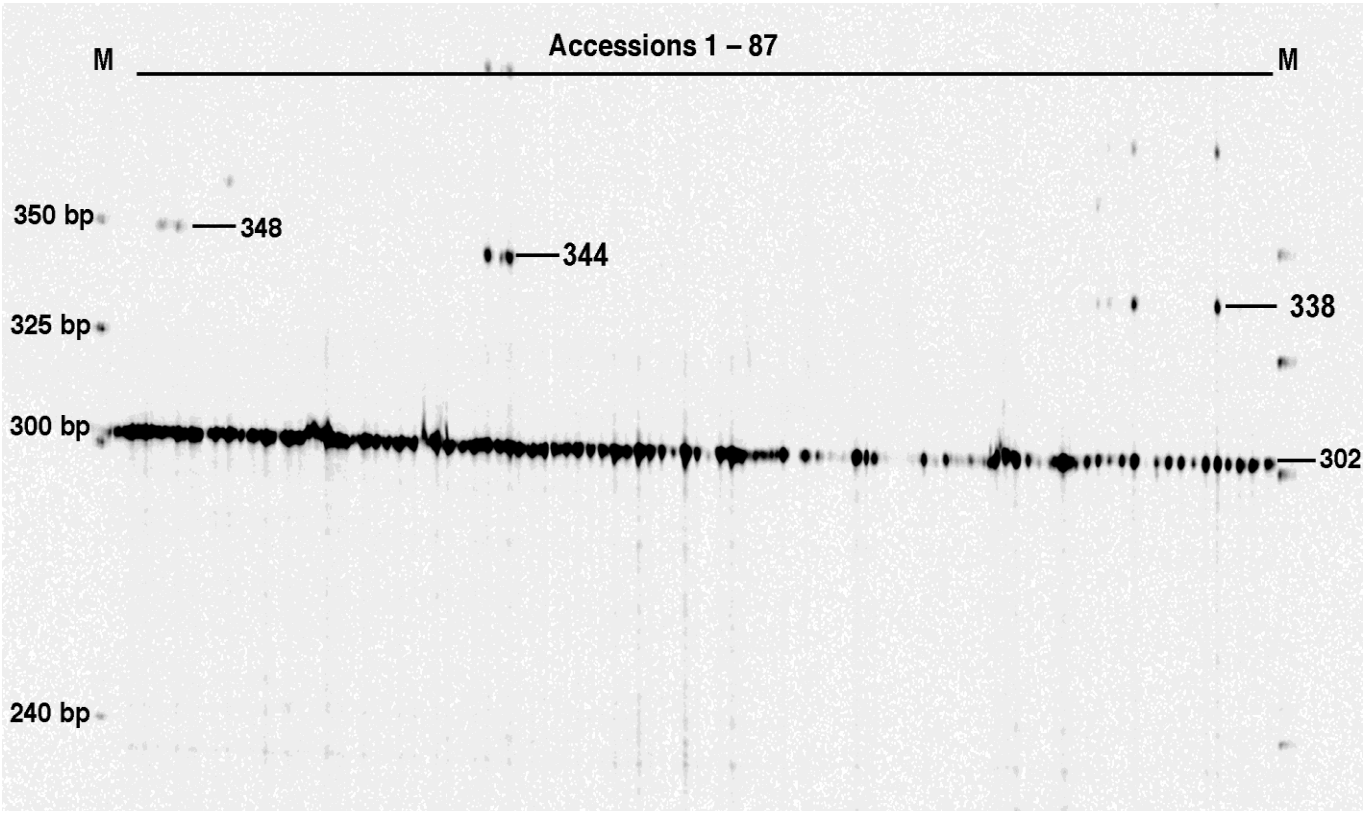

Figure 2. The maximal number of amplified loci considering all the species analyzed for each tribe. An outline phylogenetic tree representing the relationships between the seven tribes is shown on the left, adapted from Funk *et al.* (2009).