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Isolation and characterization of microsatellite markers for a worldwide invasive weed, *Chromolaena odorata* (Asteraceae)[1]

Xiang-Qin Yu[2,3,4] and Qiao-Ming Li[2,3,5]

[2]Laboratory of Plant Phylogenetics and Conservation Biology, Xishuangbanna Tropical Botanical Garden, Chinese Academy of Sciences, Kunming, Yunnan 650223, China; [3]Key Laboratory of Tropical Forest Ecology, Xishuangbanna Tropical Botanical Garden, Chinese Academy of Sciences, Menglun, Yunnan 666303, China; and [4]The Graduate School of the Chinese Academy of Sciences, Beijing 100049, China

- *Premise of the study:* Microsatellite loci were isolated and characterized for a worldwide invasive weed *Chromolaena odorata* (Asteraceae) to elucidate the population genetic structure and invasive history.
- *Methods and Results:* A total of 14 microsatellite primer pairs were developed using the Fast Isolation by AFLP of Sequences Containing repeats (FIASCO) protocol, and their polymorphism was assessed in two natural populations of *C. odorata* from Mexico and Trinidad and Tobago. Eleven loci showed polymorphism and eight of these loci were successfully amplified in *Ageratina adenophora*, another invasive weed related to *C. odorata*.
- *Conclusions:* These microsatellite markers are useful for investigating the population genetic structure and the history of range expansion of these invasive species.

Key words: *Chromolaena odorata*; invasive history; invasive weed; microsatellites; population genetics.

Chromolaena odorata (L.) R. M. King & H. Rob., native to Central and South America, is a perennial invasive weed of the family Asteraceae (Ye et al., 2004). Recently, it has spread into most of the humid tropical and subtropical regions of Africa, Asia, Oceania, and Micronesia (Muniappan et al., 2005). *Chromolaena odorata* has been listed as one of the world's 100 worst invasive alien species (Lowe et al., 2000), and it invades and spreads rapidly in open areas such as pastures, roadsides, riverbanks, disturbed forests, plantation crops, nature reserves, and wildlife sanctuaries (Lai et al., 2006), posing a serious threat to indigenous vegetation and biodiversity conservation. Several biological control programs have been implemented for controlling *C. odorata* (Muniappan et al., 2005). To plan better biological control strategies, a thorough understanding of the genetic structure, gene flow, and invasive history of *C. odorata* is needed. Here we report the development of 14 microsatellite markers to be used for population genetic studies of this species.

METHODS AND RESULTS

Total genomic DNA was extracted from leaf tissues of *C. odorata* using a modified cetyltrimethyl ammonium bromide (CTAB) method (Doyle and Doyle, 1987). Two libraries enriched for $(AC)_{15}$ and $(AG)_{15}$ repeats were constructed using the Fast Isolation by AFLP of Sequences Containing repeats (FIASCO) protocol (Zane et al., 2002) with some modifications.

About 250 ng genomic DNA was digested with the restriction enzyme *Mse*I (New England BioLabs, Ipswich, Massachusetts, USA) in a 25 μL volume, and then 15 μL of digested DNA (200–1000 bp) was ligated to an *Mse*I adaptor (5′-TACTCAGGACTCAT-3′/5′-GACGATGAGTCCTGAG-3′) using T4 DNA ligase (Fermentas, Burlington, Ontario, Canada) in a total volume of 30 μL. The ligation product was diluted (1:10) and PCR amplified with adaptor-specific *Mse*I-N primers (5′-GATGAGTCCTGAGTAAN-3′) in 20 μL volume, using a thermal cycling program of initial denaturation of 3 min at 95°C, followed by 20 cycles of 30 s at 94°C, 1 min at 53°C, 60 s at 72°C, and a final extension at 72°C for 10 min. Approximately 500 ng of amplified product was mixed with 250 pM of 5′-biotinylated $(AC)_{15}$ or $(AG)_{15}$ probe in 250 μL hybridization buffer containing 20× SSC and 10% SDS. The mixture was heated to 95°C for 5 min and incubated at 48°C for 2 h. Hybridization products and 600 μL TEN_{100} (10 mM Tris–HCl, 1 mM EDTA, 100 mM NaCl, pH 7.5) were then mixed with Streptavidin MagneSphere Paramagnetic Particles (Promega, Madison, Wisconsin, USA), which were prepared by washing three times with 150 μL TEN_{100}. The mixture was incubated at room temperature for 50 min with constant gentle agitation, three times of nonstringent and stringent washing were carried out following Zane et al. (2002), and a final washing was performed in the buffer preheated to 55°C. Recovered DNA fragments were amplified with *Mse*I-N primers. After purification using the Qiagen QIAquick PCR Purification Kit (QIAGEN, Valencia, California, USA), amplified products were ligated into pGEM-T vector (Promega) and then transformed into $DH_5\alpha$ competent cells (Takara, Dalian, Liaoning, China). The transformed cells were plated and incubated at 37°C for 12 h in Luria–Bertani culture medium. Repeat-containing clones were detected by PCR amplification using SP_6 (5′-TATTTAGGTGACACTATAG-3′)/T_7 (5′-TAATACGACTCACTATAGGG-3′) and AC_{10}/AG_{10} as primers, respectively; the thermal cycling conditions included initial denaturation of 3 min at 95°C, followed by 35 cycles of 30 s at 94°C, 30 s at 55°C, 60 s at 72°C, and a final extension at 72°C for 10 min. Positive clones were sequenced on an ABI 3730 DNA Sequence Analyzer (Applied Biosystems, Foster City, California, USA) in a volume of 5 μL including 10–20 ng of template DNA, 1.5 μL Big Dye Terminator (version 3.1), and 1 μL primer T_7 (1 pM).

A total of 54 (45%) sequences contained microsatellites with more than six repetitive units, and 45 of these sequences were selected for designing primers using the PRIMER 5.0 software package (Clarke and Gorley, 2001) and soft

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[5]Author for correspondence: lqm@xtbg.ac.cn

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TABLE 1. Characteristics of 14 identified microsatellite loci for *Chromolaena odorata*. Each forward primer was labeled with one of the three fluorescent dyes (FAM, HEX, TAMRA).

Locus	Primer sequence (5′–3′)	Repeat motif	Fluorescent dye used	T_a (°C)	No. of cycles	Size range (bp)	GenBank Accession No.
CO15	F: GTGGGTGTTACCTCTTGG R: ATCGGACATAATTTCTCCTC	$(TG)_7$	TAMRA	64	38	184–200	HM137035
CO26	F: CAGACTGGATCATAAGAA R: TTACGTGTAATAGAGCCT	$(TG)_8…(TG)_3$	TAMRA	58	40	265–271	HM137036
CO50	F: TACCCTGTTATTCCCACT R: CCTAAGCCTTCTTATTTGAT	$(TG)_{10}$	FAM	60	40	283–311	HM137037
CO56	F: ACTGGTTGGGTTTCAATG R: CTGCGTATAAAAGCGAAT	$(GT)_7$	HEX	58	38	128–132	HM137038
CO65	F: CAGTTATCTTCAACACCCAA R: TTTCCGACTAAACCCATC	$(CT)_7…(CT)_4…(TC)_3$	TAMRA	58	38	266–290	HM137039
CO77	F: TTACCGAACGTATGTTAC R: ATGCTTCATTCTTATCCC	$(TG)_{10}$	HEX	50	40	141–143	HQ913181
CO94	F: CCACGTCAAGTCCATGAT R: TCTCGATCTCAGAACGCA	$(TG)_9…(GA)_4$	FAM	54	40	110	HQ913182
CO115	F: TCGTGGTAGAGCAGAAGA R: AACTGCCAGATCAGGTTG	$(AG)_6GTT(AG)_4$	FAM	54	38	312–346	HM137040
CO156	F: CGTACCACGTCAAGTCCA R: TCTCGATCTCAGAACGCA	$(TG)_9…(GA)_4$	FAM	52	40	113	HQ913183
CO165	F: TTGATCTCGGCTTAGAAT R: TGGTATAAATCGGTATGG	$(GT)_7$	FAM	52	40	109	HM137041
CO189	F: AGAGTAAGCACGAGACCG R: AGAACTTTACCTCCCACA	$(TTTTG)_3…(AG)_9$	FAM	60	38	159–173	HM137042
CO195	F: AAGAATGCACAAAATCAG R: CTTTCAGTCTCAGACGAA	$(GATT)_3…(TG)_8$	HEX	56	40	183–187	HM137043
CO227	F: GTTCGTCACCCTTTTCTC R: ATCTGCACTTCATCTTCTTC	$(GA)_5…(AG)_9$	HEX	62	40	193–219	HM137044
CO250	F: AAGGACCTCTACCTATCA R: ATTTCTGCCCATCTTATT	$(CA)_{15}$	FAM	58	40	79–95	HQ913184

T_a, annealing temperature.

OLIGO 6 (National Biosciences Inc., Plymouth, Minnesota, USA). These primers were tested for polymorphism in 42 individuals of *C. odorata*. PCR amplification was performed using an ABI Gene Amp 9700 PCR system in a volume of 20 µL containing 2.5 µL of 10× buffer, 0.2 mM of each dNTP, 2.0 mM of $MgCl_2$, 0.4 µM of each primer, 1 U of *Taq* polymerase (Takara), and 20 ng of genomic DNA. The amplification conditions included an initial denaturing at 95°C for 5 min, followed by 35 to 40 cycles of 40 s at 94°C, 45 s at the locus-specific annealing temperature (T_a in Table 1) and 45 s at 72°C, and then a final extension for 10 min at 72°C. Each forward primer was labeled with one of the three fluorescent dyes (FAM, HEX, TAMRA) for polymorphism analysis on an ABI 3730xl DNA analyzer (Applied Biosystems), using an internal lane standard Rox-500 (Microread Genetics, Beijing, China). PCR products with different sizes were multiplexed for detecting, and each mixture contained products of two to three primers labeled with different fluorescent dyes.

Thirty individuals from Mexico (Veracruz, Teocelo city: 19°23′N, 96°58′W, 1160 m elevation) and 12 from Trinidad and Tobago (Borough of Chaguanas, Felicity city: 10°31′N, 61°25′W, 10 m elevation) were used for genotyping. Fourteen of 45 primers amplified PCR products of expected size range with a single clear band, 11 primer pairs showed polymorphism, and the remaining three showed monomorphism. Sequences of 14 loci (accession numbers HM137035–HM137044 and HQ913181–HQ913184) were deposited in GenBank (Table 1). Population genetic analyses of these loci were performed using both GENALEX (Peakall and Smouse, 2006) and GENEPOP version 3.4 software (Raymond and Rousset, 1995). The number of alleles per locus ranged from one to 13, with a mean of 4.714. The observed heterozygosity (H_o) per locus ranged from 0.000 and 1.000, and expected heterozygosity (H_e) ranged from 0.000 and 0.862, respectively (Table 2). No significant linkage disequilibrium was detected between pairs of loci.

For cross-species amplification, we chose *Ageratina adenophora* (Spreng.) R. M. King & H. Rob., another worldwide invasive species (*C. odorata* and *A. adenophora* were both formerly treated in genus *Eupatorium*). All of the 14 loci were tested in 10 individuals of *A. adenophora* collected from China (Yunnan Province, Kunming city: 24°58′N, 102°37′E, 1932 m elevation). Eight of the 14 loci were successfully amplified and produced expected product sizes (Table 3), and six loci including CO165, which is monomorphic in 42 individuals of *C. odorata*, showed polymorphism.

CONCLUSIONS

The 11 polymorphic microsatellite loci presented here are useful for investigating the population genetic structure of *C. odorata* and tracing its invasive history to develop suitable strategies for the management of this invasive weed. We are presently using several of these loci to analyze the genetic

TABLE 2. Results of initial primer screening in 42 individuals of *Chromolaena odorata* for 14 loci.[a]

	Mexico (N = 30)			Trinidad and Tobago (N = 12)		
Locus	A	H_o	H_e	A	H_o	H_e
CO15	1	0.000	0.000	4	1.000	0.576
CO26	4	0.767	0.713	2	0.000	0.153
CO50	8	0.600	0.715	3	0.250	0.288
CO56	3	0.400	0.456	1	0.000	0.000
CO65	5	0.000	0.556	4	0.917	0.649
CO77	2	0.000	0.320	2	0.000	0.500
CO94	1	0.000	0.000	1	0.000	0.000
CO115	4	0.367	0.569	2	0.000	0.153
CO156	1	0.000	0.000	1	0.000	0.000
CO165	1	0.000	0.000	1	0.000	0.000
CO189	7	0.733	0.744	4	0.917	0.740
CO195	2	0.000	0.064	2	0.917	0.497
CO227	13	0.379	0.862	3	0.167	0.403
CO250	4	1.000	0.547	1	0.000	0.000

A, number of alleles; H_e, expected heterozygosity; H_o, observed heterozygosity.

[a] Voucher specimens of *C. odorata* (Teocelo population: 135941; Felicity population: 135944) were deposited at the herbarium of the Xishuangbanna Tropical Botanical Garden (HITBC), Chinese Academy of Sciences.

TABLE 3. Cross-amplification of eight microsatellite loci across *Ageratina adenophora*.[a]

Locus	No. of successfully amplified individuals	No. of alleles (size)	H_o	H_e
CO50	2	2 (285, 289)	0.000	0.500
CO56	5	3 (128, 130, 132)	0.600	0.460
CO77	7	1 (143)	0.000	0.000
CO156	3	1 (113)	0.000	0.000
CO165	10	3 (105, 107, 109)	0.200	0.580
CO189	8	5 (159, 163, 165, 167, 169)	0.375	0.672
CO227	3	3 (203, 207, 219)	0.000	0.667
CO250	10	2 (87, 89)	1.000	0.500

H_e, expected heterozygosity; H_o, observed heterozygosity.

[a] A voucher specimen of *A. adenophora* (135935) was deposited at the herbarium of the Xishuangbanna Tropical Botanical Garden (HITBC), Chinese Academy of Sciences.

structure of *C. odorata* in its native (e.g., Mexico, Puerto Rico, Cuba) and invasive (e.g., China, India, Cambodia) regions.

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