Development of eight microsatellite markers in big sagebrush (*Artemisia tridentata* Nutt.)

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Running Title: Microsatellite markers of *A. tridentata*

19 **Abstract**

20 We developed eight polymorphic microsatellite (SSR) loci from genomic DNA of big
21 sagebrush *Artemisia tridentata* Nutt. using a dual suppression-PCR technique and an
22 improved technique. These markers with four to eighteen alleles per locus successfully
23 distinguished each individual collected from a population in California, USA. The
24 observed and expected heterozygosities raged from 0.045 to 0.850 and 0.194 to 0.897,
25 respectively. These SSR markers will be available to identify genets and evaluate
26 genetic relationship between individuals of *A. tridentata*.

27 Big sagebrush (*Artemisia tridentata* Nutt.) is the dominant plant of the Great Basin
28 region of Western North America (McArthur & Sanderson 1999). *Artemisia tridentata*
29 has an extensive list of herbivores (Wiens *et al.* 1991) including vertebrates and insects
30 of almost every feeding guild. *Artemisia tridentata* induces its own systemic resistance
31 via airborne volatiles which are released from damaged leaves (Karban *et al.* 2006). In
32 addition, these volatiles also induce systemic resistance of intact neighboring plant
33 growing up to 60cm apart from the damaged individual in the field (Karban *et al.*
34 2006), which is referred to as volatile communication. The induction of resistance in
35 neighboring plant would appear to benefit to the neighboring plant by reducing
36 herbivory, that is, volatile communication may be altruistic. Therefore, to evaluate the
37 ecological and evolutionary impact of volatile communication, it is important to reveal
38 the genetic relationship between plants that emit the volatiles and plants that receive
39 those. Thus, we isolated microsatellite markers from *A. tridentata* to investigate the
40 genetic relationship between individuals growing in natural population.
41 Total DNA was extracted from fresh leaves of *A. tridentata* with
42 cetyltrimethyl ammonium bromide (CTAB) method (Stewart & Via 1993). We isolated
43 microsatellites using a dual-suppression-polymerase chain reaction (PCR) method
44 (Lian & Hogetsu 2002) and an improved technique (Lian *et al.* 2006). Briefly, in order
45 to construct adaptor ligated DNA libraries, DNA was digested separately using the
46 restriction enzymes *Afa* I, *Alu* I, *Eco*R V, *Hae* III, and *Ssp* I. The fragments produced
47 by each restriction enzyme were then ligated to an unequal length adaptor (see, Lian &
48 Hogetsu 2002), using a DNA Ligation Kit version 2.1 (Takara). The DNA fragments
49 with a microsatellite at one end were amplified from the *Afa* I DNA library using SSR
50 primer $(AC)_6(AG)_5$, $(AG)_6(AC)_5$, $(AC)_6(TC)_5$ or $(TC)_6(AC)_5$ and an adaptor primer

51 AP2 (5'-CTATAGGGCACGCGTGGT-3') according to Lian *et al.* (2006). The
52 fragments amplified by $(AC)_6(TC)_5$ and $(TC)_6(AC)_5$ were purified by QIAquick PCR
53 Purification Kit (QIAGEN). The purified fragments were cloned into pDrive Cloning
54 Vector and the plasmids were transformed into QIAGEN EZ Competent Cells, using
55 PCR Cloning^{plus} Kit (QIAGEN). The cloned fragments were amplified using the M4
56 forward and M13 reverse primers from the plasmid DNA of positive clones. These
57 products were purified as described above, and then, sequenced directly using the
58 BigDye Terminator Cycle Sequencing Kit (Applied Biosystems) with the ABI PRISM
59 3100 automated sequencer (Applied Biosystems).
60 For each fragment amplified by the $(AC)_6(TC)_5$ or $(TC)_6(AC)_5$ primer, a
61 locus-specific primer (IP1) was designed from the sequenced region flanking the SSR.
62 The primer pairs of IP1 primer and SSR primer were used as a SSR marker (Lian *et al.*
63 2006).
64 To investigate microsatellite polymorphism, 40 leaves collected from *A.*
65 *tridentata* population at Sagehen Creek, north of Truckee, California, USA were used.
66 PCR amplification was carried out with the GeneAmp PCR system 9700 thermal
67 cycler (Applied Biosystems) in a reaction mixture (10 μL) containing 5 ng of template
68 DNA, 0.2 mM of each dNTP (Applied Biosystems), 10 x PCR buffer with 1.5 mM
69 $MgCl_2$ (Applied Biosystems), 0.25 U of Ampli *Taq* Gold DNA polymerase (Applied
70 Biosystems), and two primers: IP1 (0.5 μM) and the SSR primer (fluorescent
71 dye-labeled, 0.5 μM). The PCR condition was 9 min at 94 °C, 35 cycles of 30 sec at
72 94 °C, 30 sec at the annealing temperature and 1 min at 72 °C, followed by 72 °C for 7
73 min. The PCR products were detected with an ABI PRISM 3100 automated sequencer
74 (Applied Biosystems).

75 We successfully identified eight SSR loci with polymorphism and
76 codominance from *A. tridentata* (Table 1). Forty genotypes were detected in 40
77 samples analyzed by these markers. The number of alleles per locus ranged from four
78 to 18 per locus, with an average of 7.63 (Table 1). The observed and expected
79 heterozygosities calculated using CERVUS version 3.0.3 (Kalinowski *et al.* 2007)
80 ranged from 0.045 to 0.850 and 0.194 to 0.897, respectively. Statistical tests for
81 Hardy-Weinberg equilibrium and linkage disequilibrium were conducted with
82 GENEPOP version 3.4 (Raymond & Rousset 1995) using default values for Markov
83 chain parameters. We also estimated the frequency of null alleles using Brookfield I
84 method (2000 permutation) implemented in the program MICRO-CHECKER version
85 2.2.3 (Van Oosterhout *et al.* 2004). Three of eight loci showed significant deviation
86 from Hardy-Weinberg equilibrium (HWE) after Bonferroni correction (overall α =
87 0.01) due to heterozygote deficiency (Table 1). Because the three loci exhibited high
88 levels of null allele frequencies (0.197, 0.278 and 0.377 for AT4, AT13 and AT25,
89 respectively; $\alpha = 0.05$), we assume that presence of null alleles are responsible for the
90 deviation from HWE. There were no significant linkage disequilibriums between the
91 eight loci ($\alpha = 0.05$). These markers are currently being used to analyze the genetic
92 relationship between *A. tridentata* individuals growing in natural population in
93 California, USA.
94

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Table 1 Characteristics of eight microsatellite loci from *A. tridentata*.

Locus	Repeat	Primer sequence (5' ~ 3') and fluorescent label	T_a (°C)	Size range (bp)	No. of alleles	H_O	H_E	GeneBank Accession no.
AT2	$(TC)_6(AC)_7$	TCTCTCTCTCTCACACACACAC	57	100-105	4	0.513	0.611	AB488553
		(NED)CTTGCCCATTGTGATTATTTTGAC						
AT4	$(TC)_6(AC)_5$	TCTCTCTCTCTCACACACACAC	60	104-116	10	0.432	0.794*	AB488554
	$...(AC)_4$	(VIC)AAGCATTGGCAAAACGTGAGATTA						
AT13	$(AC)_6(TC)_9$	ACACACACACACTCTCTCTCTC	57	293-356	18	0.361	0.897*	AB488555
		(NED)TTCAACGAGTTTCACTCCAGTTTA						
AT15	$(AC)_6(TC)_5$	ACACACACACACTCTCTCTCTC	60	102-121	8	0.769	0.790	AB488556
	$...(AC)_{13}...(TC)_3$	(PET)GGTACTACTAGCTAACAGTTGAAT						
AT16	$(AC)_6(TC)_9$	ACACACACACACTCTCTCTCTC	58	138-158	12	0.850	0.846	AB488557
		(VIC)TAAGAAGCTCTTGCTTTGAAGCTC						
AT22	$(TC)_6(AC)_5T$-	TCTCTCTCTCTCACACACACAC	58	138-153	9	0.525	0.641	AB488558
	$(AC)_2T(AC)_3$	(FAM)ACATAAGGGCCACAAAACAAAGTC						
AT25	$(AC)_6(TC)_6$	ACACACACACACTCTCTCTCTC	60	160-168	5	0.045	0.692*	AB488559
	$...(AC)_4$	(FAM)TAGTGGAGCGTTTCTTGAACTCTG						
AT28	$(AC)_6(TC)_6$	ACACACACACACTCTCTCTCTC	58	244-255	5	0.103	0.194	AB488560
	$...(CA)_4...(AC)_3$	(VIC)GATAACGATTCAACTCGGTCAACA						

T_a, annealing temperature of the primer pair; H_O, observed heterozygosity; H_E, expected heterozygosity.

* indicates significant deviation from Hardy-Weinberg equilibrium after Bonferroni correction (adjusted $P < 0.0013$)