

Microsatellite primers in *Plantago virginica* (Plantaginaceae), an invasive species with both cleistogamous and chasmogamous flowers

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Polymorphic microsatellite markers were developed in *Plantago virginica*, an invasive species in China with both cleistogamous and chasmogamous flowers, to investigate its genetic structure and mating patterns. Fourteen novel microsatellite primer sets were designed, and the marker loci they amplified were characterized in 96 individuals from four populations. Eleven of these markers showed polymorphism and the number of alleles per locus ranged from two to six. AMOVA and STRUCTURE indicated that there were distinct patterns of genetic differentiation among the one invasive and three native US populations. These markers provide a useful tool for investigating genetic diversity in *P. virginica* and studying the mechanisms of invasion success.

Key words: microsatellite, invasive species, cleistogamy, *Plantago virginica*, genetic structure

INTRODUCTION

The mating system of flowering plants influences important population genetic parameters, including genetic recombination, effective population size, gene flow and the partitioning of genetic diversity within and among populations, and plays a key role in determining the success of species' range expansion or invasion (Barrett et al., 2008). Some species with a natural mixed mating system including both cleistogamous (CL) (permanently closed, self-pollinated, i.e., selfing) and chasmogamous (CH) (wind- or insect-pollinated, i.e., outcrossing) flowers are regarded as ideal models for scientists to study the ecological, developmental and evolutionary aspects of plant reproduction. Although research on cleistogamy will likely provide new insight into the evolutionary history and prevalence of this mating system, very few studies have focused on species that reproduce through both CH and CL flowers (Culley and Wolfe, 2001). In particular, the precise role of the mating system of cleistogamy in invasion success is still poorly understood, and future comparative and experimental studies are desirable.

Plantago virginica, an alien weed from North America,

was first reported in China in the 1950s, and has extensively invaded farmland, lawns and forests in eastern and southern China (Guo et al., 1996). This study focused on *P. virginica* not only because of its high invasiveness, but also because it possesses a naturally mixed mating system (CH & CL). Analyses of the genetic structure and diversity of *P. virginica* will help us to characterize the extent of differentiation among populations, ecotypes, forms and subspecies, and its molecular evolution. Therefore, we first developed and characterized 11 polymorphic microsatellite (simple sequence repeat, SSR) primers for *P. virginica*, to enable further population genetic analyses.

MATERIALS AND METHODS

Isolation of microsatellite loci Microsatellite makers were developed using samples from four *P. virginica* populations (each comprising 24 individuals), including three natives collected from Logan County (OK), Statesboro (GA) and Charlottesville (VA), USA, and one invasive from Nanchang (Jiangxi), China. Extracted genomic DNA of *P. virginica* was digested with *Rsa*I and *Xmn*I and the resulting fragments were ligated to SuperSNX24 linkers (Glenn and Schable, 2005). The ligation products with potential microsatellite loci were identified by hybridization with the 5' biotinylated oligonucleotides (CT)₁₃, (AAG)₈, (AGG)₆, (CCG)₆, (ATC)₆ and (AGC)₆. Microsatel-

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lite enrichment was completed by streptavidin magnetic beads, and captured DNA was recovered by polymerase chain reaction (PCR) with SuperSNX24F adaptor-specific primers. The fragments enriched with microsatellite loci were cloned using pMD18-T vector and transformation of *Escherichia coli* competent cells (DH5 α , Takara). M13 primers were used for PCR amplification to isolate the recombinant clones, and positive clones were then sequenced using an ABI 377XL DNA Sequencer. Primers for each microsatellite were designed by Primer Premier 5.0, according to the following three criteria: the primers are 18–22 bp in length; the annealing temperature is 5 °C lower than the T_m value (55–65 °C); and the GC content is 35–60%.

Characterization of microsatellite markers A total of 300 positive clones containing repeat regions were

found, and 40 of these were suitable for locus-specific design. Fourteen of these primers with annealing temperatures ranging from 59 °C to 64 °C amplified single marker loci successfully (Table 1), and the markers were then tested for polymorphism in four populations (24 individuals in each population). The PCR amplifications were performed in a 10- μ l reaction mixture and the PCR program consisted of 5 min at 95 °C, 35 cycles of 40 s at 94 °C, 50 s at the primer-specific temperature (Table 1), 1 min at 72 °C, and a final step of 8 min at 72 °C. Markers with polymorphism were screened by 6% polyacrylamide gel electrophoresis, and fragment sizes were estimated using pUC19 DNA/MspI (HpaII) Marker.

The software GenAlEx 6.5 was used to calculate the number of alleles per locus (N_A), observed (H_O) and expected heterozygosity (H_E) and inbreeding coefficient (F_{IS}), and to test the Hardy-Weinberg equilibrium

Table 1. Characteristics of 14 microsatellite primers designed in *P. virginica*

Primer	Sequence	Repeat	Size (bp)	Ta (°C)	GenBank Accession No.
PV1	F: TTCTTCATCAAACCTCTGCTA R: TTGAAACAGGAAATGAGC	(TC) ₆	207–209	58	KP714717
PV3	F: CCATTCCCAGAAACCAGC R: CGACGAGTGGCGAGTAGA	(CT) ₅	203	56	KP714718
PV4	F: TGATGAGTTCAGGTGGTC R: ATGTGACTGGGAAGGAG	(CT) ₃	181	62	KP714719
PV8	F: TCTCCCATCTCACTTCACC R: ATCCGGTACGGCTATTGT	(TC) ₄	266–291	62	KP714720
PV10	F: TGAGTTGGTTTGGCAGAG R: GGAAGAAGTGGGAGGACA	(GA) ₈	222–275	62	KP714721
PV11	F: GCAATCCCCAAGAACAT R: CATAGTTTATTACGAGGTGG	(TA) ₇	329–373	60	KP714722
PV12	F: GTGCTCCGGTTTGCTCT R: TCCACGGAACCAACATC	(TC) ₅	300	64	KP714723
PV13	F: TAACCACCGAATTACGCC R: GGAGTCAGACTTAGACGCAA	(TG) ₃	263–365	63	KP714724
PV14	F: AGTGAAGGACGAACAACC R: CTTCTTCCGCAATACACT	(AAC) ₅	248	61	KP714725
PV15	F: AGGATGCCGAGGGTATTT R: GACAAAGCAAACCCCTCATT	(AGTT) ₈	310–324	61	KP714726
PV16	F: AAAGAAGTTGGTTCCGAGTG R: GAATGAAGGCGGTGGAAA	(CT) ₁₀	304–351	64	KP714727
PV26	F: CAACATAAGCCAAGTGGTAGA R: TGTGGCATTATCCCTTCA	(TC) ₃	176	62	KP714728
PV39	F: TCTGGCATGAACTGGTGT R: AAGTTAGCCTCGGTTGGA	(AG) ₉	217–224	61	KP714729
PV40	F: GCGAGCATTGGCTTTCTA R: AAGTTAGCCTCGGTTGGA	(AG) ₉	108–133	61	KP714730

(HWE) (Peakall and Smouse, 2012). MICRO-CHECKER detected no null alleles (Van Oosterhout et al., 2004).

Genetic structure analysis To infer the genetic structure, assignments of individuals to populations were made with STRUCTURE ver. 2.3.2 (Pritchard et al., 2000), which implements a method of Bayesian-based clustering and defines the genetic clusters with no prior information of population origin. The Admixture Model was used for the STRUCTURE analysis. Ten independent runs each for $K = 1$ to 8 were performed with 50,000 Markov Chain Monte Carlo repetitions after a burn-in period of 100,000 iterations. The appropriate number of clusters (K) based on ΔK (Evanno et al., 2005) was deter-

mined using the program STRUCTURE HARVESTER (Earl and vonHoldt, 2012). The statistical significance of genetic differentiation among the studied populations and between invasive and native regions was determined by an analysis of molecular variance (AMOVA) test (with 9,999 permutations) using GenAlEx 6.5 (Peakall and Smouse, 2012).

RESULTS AND DISCUSSION

In the four *P. virginica* populations, 11 of the 14 marker loci showed polymorphism. The number of alleles varied from two to six per polymorphic locus (Table 2), and H_O and H_E ranged from 0.000 to 0.542 and 0.000 to 0.760,

Table 2. Results of primer screening for 11 polymorphic loci in four populations (three native populations and one invasive population) of *P. virginica*

Locus	Logan County (N = 24)				Statesboro (N = 24)			
	N_A	H_O	H_E	F_{IS}	N_A	H_O	H_E	F_{IS}
PV1	1	0.000	0.000	NA	1	0.000	0.000	NA
PV4	1	0.000	0.000	NA	1	0.000	0.000	NA
PV8	2	0.048	0.046	-0.024	4	0.176	0.166	-0.062
PV10	6	0.000	0.760***	1.000	2	0.000	0.497***	1.000
PV11	6	0.048	0.756***	0.937	2	0.000	0.500***	1.000
PV13	6	0.000	0.083***	1.000	1	0.000	0.000***	NA
PV14	6	0.000	0.000	NA	2	0.000	0.153***	1.000
PV15	4	0.167	0.433***	0.615	2	0.000	0.444***	1.000
PV16	5	0.167	0.688***	0.758	3	0.000	0.500***	1.000
PV39	2	0.083	0.080	-0.043	2	0.042	0.041	-0.021
PV40	4	0.042	0.437***	0.905	4	0.542	0.666***	0.186
Mean	3.091	0.050	0.298	0.643	2.182	0.069	0.270	0.638
	Charlottesville (N = 24)				Jiangxi (N = 24)			
	N_A	H_O	H_E	F_{IS}	N_A	H_O	H_E	F_{IS}
PV1	2	0.000	0.080***	1.000	1	0.000	0.000	NA
PV4	2	0.000	0.153***	1.000	1	0.000	0.000	NA
PV8	3	0.000	0.156***	1.000	3	0.043	0.232***	0.812
PV10	1	0.000	0.000	NA	2	0.000	0.080***	1.000
PV11	1	0.000	0.000	NA	1	0.000	0.000	NA
PV13	1	0.000	0.000	NA	2	0.000	0.219***	1.000
PV14	1	0.000	0.000***	NA	1	0.000	0.000	NA
PV15	1	0.000	0.080***	1.000	2	0.000	0.080***	1.000
PV16	1	0.000	0.000	NA	1	0.000	0.000	NA
PV39	3	0.000	0.526***	1.000	2	0.083	0.080	0.043
PV40	6	0.042	0.681***	0.939	2	0.000	0.486***	1.000
Mean	2.091	0.004	0.152	0.990	1.636	0.012	0.107	0.795

N_A = number of alleles per locus; H_O = observed heterozygosity; H_E = expected heterozygosity; F_{IS} = inbreeding coefficient; NA = not available because locus was monomorphic.

*** Indicates significant departure from HWE ($P < 0.001$).

respectively. All loci showed significant deviation from HWE ($P < 0.001$), and all populations had high F_{IS} (Table 2), indicating that selfing predominates in *P. virginica*, while outcrossing occurs less frequently. The population in China (Nanchang) showed lower genetic diversity than two of the native populations (Logan County and Statesboro) in all measures of diversity: mean number of alleles, H_O and H_E (1.636, 0.012 and 0.107, respectively, in Nanchang) (Table 2).

The method of Evanno et al. (2005) used in STRUCTURE HARVESTER also indicated that ΔK was at a maximum when $K = 4$ (Fig. 1). Each genetic cluster was represented largely by one of four populations in our samples (Fig. 2). Results from STRUCTURE demonstrated that there were distinct genetic differentiations between the invasive and native regions and among the four populations. The populations of Logan County and Statesboro contained elements from all four genetic clusters, while each population of Charlottesville in the US and Nanchang in China mainly came from one genetic clus-

ter (Fig. 2). Meanwhile, AMOVA also indicated that populations were partitioned into two regions (invasive vs. native), and most of the genetic variance could be explained by variation within populations (49%) (Table 3). The genetic variation between regions and among populations within regions was 23% and 29% of total genetic variation, respectively (Table 3).

Overall, we developed 11 microsatellite markers for *P. virginica* and these markers showed sufficient polymorphism to analyze the genetic diversity and structure among and within populations of *P. virginica*. Using the SSR markers developed here, we found that the invasive population exhibited lower genetic diversity than native populations. The decrease of genetic diversity may result from a larger extent of selfing, the founder effect or limited gene flow during its invasion from North America to China. Given that only one invasive population was investigated in the present study, the results reported here can provide only a preliminary explanation with respect to the mechanism of invasion in *P. virginica*. In future studies, more populations from both native and invasive regions should be investigated by employing the newly developed SSR markers to detect the genetic differentiation and its role in explaining invasiveness in *P. virginica*.

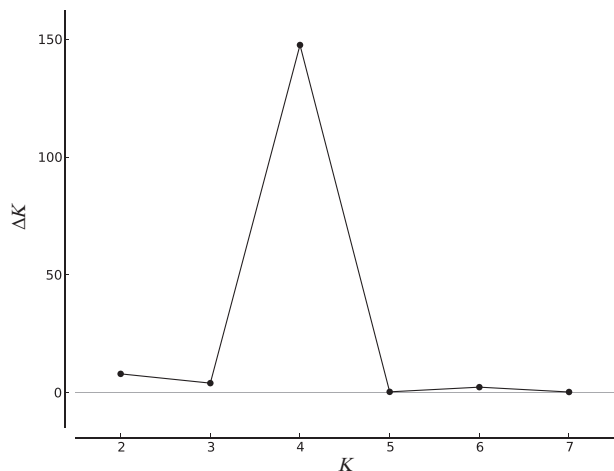


Fig. 1. Values of ΔK , based on the rate of change in $\ln P(X/K)$ between successive K values generated from STRUCTURE HARVESTER.

Table 3. Analysis of molecular variance for *P. virginica* populations with 11 microsatellite loci

Source of variation	df	Sum of squares	Variance components	% of variation
Between regions (US and China)	1	165.573	2.411	23%
Among populations within regions	2	157.583	3.067	29%
Within populations	92	476.875	5.183	49%
Total	95	800.031	10.661	100%

$F_{CT} = 0.226^{**}$, $F_{SC} = 0.372^{**}$, $F_{ST} = 0.514^{**}$
** $P < 0.01$.

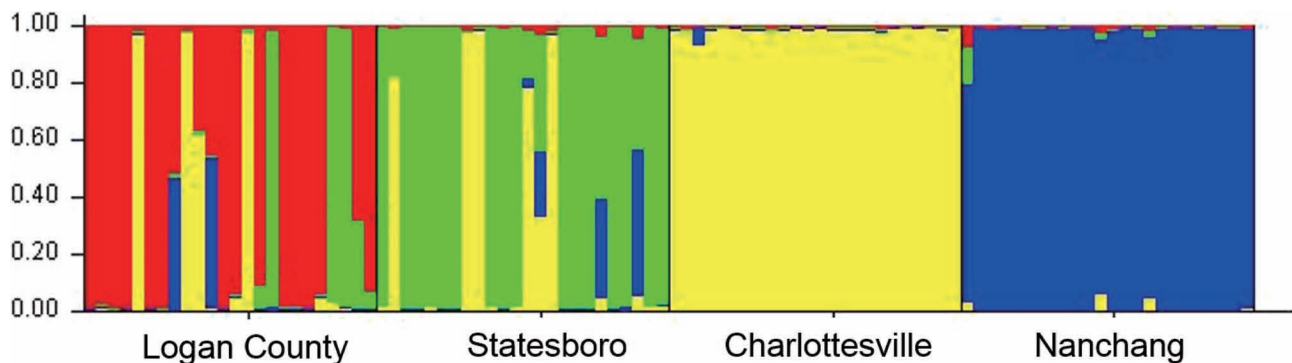


Fig. 2. Cluster analysis by STRUCTURE, based on 11 microsatellite loci for *P. virginica* populations in China (Nanchang) and the US (Logan County, Statesboro and Charlottesville).

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