Eleven Microsatellites in an Emerging Invader, Phytolacca americana (Phytolaccaceae), from Its Native and Introduced Ranges

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Source: Applications in Plant Sciences, 3(3)

Published By: Botanical Society of America

URL: https://doi.org/10.3732/apps.1500002

Applications in Plant Sciences 2015 3(3): 1500002

PRIMER NOTE

ELEVEN MICROSATELLITES IN AN EMERGING INVADER, *PHYTOLACCA AMERICANA* (PHYTOLACCACEAE), FROM ITS NATIVE AND INTRODUCED RANGES[1]

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- *Premise of the study:* To facilitate population genetic analyses, microsatellite markers were developed for pokeweed (*Phytolacca americana*), a large, weedy, perennial herb native to eastern North America that is emerging as a significant invasive species in China.
- *Methods and Results:* We mined 1,100,538 Illumina MiSeq reads from genomic DNA for microsatellites and identified 58 primer pairs. We screened these primers for polymorphism in two native and two invasive populations. We identified 11 loci that amplified consistently. The number of alleles per locus ranged from two to six, and observed heterozygosity ranged from 0.00 to 1.00. All loci were largely monomorphic within populations but different among populations. The primers were of very limited use in the congener *P. acinosa*.
- *Conclusions:* These loci will provide a valuable resource to study the population genetics and invasion history of *P. americana*.

Key words: biological invasions; Illumina; MiSeq; *Phytolacca americana*; Phytolaccaceae; pokeweed.

Pokeweed, *Phytolacca americana* L. (Phytolaccaceae), is a large, weedy, perennial herb native to eastern North America but widely distributed in Asia and Europe. With high seed production and bird-dispersed fruits, the plant establishes readily in disturbed habitats (McDonnell et al., 1984). Pokeweed was likely intentionally introduced to China and first reported in 1935 in Zhejiang Province (Li and Xie, 2002; Xu et al., 2012). Recent reports in Asia indicate that *P. americana* may have emerged as a more aggressively invasive species where it has established (Kim et al., 2005). The species has recently become a significant threat to coastal forest ecosystems in China (Zhai et al., 2010; Fu et al., 2012). Furthermore, *P. americana* has largely displaced a Chinese native congener, *P. acinosa* Roxb., in parts of China. Although *P. acinosa* has been a historic part of Chinese pharmacopoeias for more than 2000 years, it is similar in appearance to *P. americana*, which in turn is considerably more toxic than *P. acinosa* (Kim et al., 2005), leading to the possibility of accidental poisonings. Despite its widespread distribution and emergence as an invasive species, many aspects of pokeweed evolutionary ecology are not well understood, including its breeding system. The microsatellites we have developed could be valuable tools to understand pokeweed population structure and invasion history.

METHODS AND RESULTS

We extracted genomic DNA from freshly collected leaves of one individual of *P. americana* from Memphis, Tennessee, using a QIAGEN DNeasy Plant Mini Kit (QIAGEN, Valencia, California, USA). We mined 1,100,538 Illumina MiSeq reads from genomic DNA from that individual for microsatellites and flanking primers using the PAL Finder pipeline (Castoe et al., 2012). We added a CAG sequence to the 5′ end of one of each of the primer pairs to facilitate use of a third, fluorescently labeled primer in the PCR.

To screen the primers, we collected leaf samples from populations both in the native and introduced range of *P. americana*: Florida, USA; Illinois, USA; Anhui, China; and Fukushima, Japan. We also collected leaf samples from a single population of the native *P. acinosa* in Jiangsu, China (GPS coordinates 32.06°N, 118.83°E). Collection coordinates and voucher information are provided in Appendix 1. Leaves were dried, frozen, and lysed before genomic DNA was extracted as described above. We tested 58 primer pairs for amplification; 11 produced consistent results for *P. americana* (Table 1). We were able to amplify only a single locus (PW65) for *P. acinosa*, where 12 of the 21 samples yielded products in the expected size range given data from *P. americana*. We amplified each locus in a 12.5-μL PCR reaction (10 mM Tris-HCl, 50 mM KCl, 0.1% Triton X-100, 100 μg/mL bovine serum albumin [BSA], 0.4 μM unlabeled primer, 0.04 μM tag-labeled primer, 0.36 μM universal dye-labeled primer, 4.0 mM $MgCl_2$, 0.8 mM dNTPs, 0.25 units EconoTaq Polymerase [Lucigen, Middleton, Wisconsin, USA], and 10 ng DNA template) using a touchdown protocol where the annealing temperature decreased by 0.5°C for each of the first 20 cycles: 96°C (2.5 min), then 20 cycles of 96°C (30 s), initial 65°C (30 s), 72°C (30 s), then 20 cycles: 96°C (30 s), 50°C (30 s), 72°C (30 s), then 72°C (10 min). Initial annealing temperature was 60°C for PW182. After PCR, amplified fragments were diluted 1 : 5 and pooled in the following four groups: PW106, PW29, and PW43; PW54, PW46, and PW79; PW69, PW11,

[1]Manuscript received 4 January 2015; revision accepted 30 January 2015.

The authors thank M. M. Mauricio and H. A. Dahn for collecting field samples, H. Donaldson and M. Marks for assistance in screening primers, U. Bagal for help in analyzing data, and an anonymous reviewer for comments. This work was supported by the National Science Foundation Partnership for International Research and Education (PIRE) program (OISE 0730218). K.E.B. was supported by a National Science Foundation Graduate Research Fellowship (DGE0903734).

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doi:10.3732/apps.1500002

TABLE 1. Characteristics of 11 microsatellite loci developed in *Phytolacca americana*.

Locus	Primer sequences (5′–3′)	Repeat motif	Allele size range (bp)[a]	T_a (°C)	Fluorescent dye[b]	GenBank accession no.
PW11	F: AGCCGGAGGTCTCTCTTGG	$(AAC)_{33}$	354–372	65–55	FAM	KP331491
	R: *CAGTTTAGAAATCTGGAATTAGAGTTGG					
PW29	F: *GATGAAGAAAGGGCAACCCC	$(AATAAG)_{30}$	354–366	65–55	HEX	KP331492
	R: ACGAGTGCAGATCCAAGTGC					
PW46	F: *GGATGCAAATAATCCTAGTTCGG	$(ATT)_{36}$	331–352	65–55	HEX	KP331493
	R: CAGACTCCCGAGTTTGTCCC					
PW53	F: AATCTTTGGCTTGGCCACC	$(ATAC)_{24}$	454–458	65–55	HEX	KP331494
	R: *TCAAAAGACAATGCAGAAGCC					
PW54	F: *CATATCACGAGAGGGGAGCC	$(AAAAG)_{30}$	283–298	65–55	FAM	KP331495
	R: GGTAACCTCATTGGGACCCG					
PW65	F: *CACAACAAGCGTTAATTTACGAGC	$(ATC)_{33}$	359–368	65–55	HEX	KP331496
	R: GTCATGCTCCTGCTCAGTCC					
PW69	F: *GGATGGTTTCTTCATGCAACG	$(AAAAG)_{25}$	302–312	65–55	HEX	KP331497
	R: AGCAAATCCTTGATCAGCCC					
PW79	F: *TTGCCAATAGTAACTCTTTGAACTCC	$(ATGTCC)_{36}$	387–399	65–55	FAM	KP331498
	R: CCAGAATGTGGGATTGAGGG					
PW106	F: CTAATATGAGCTTTAGCAACACTGC	$(ATT)_{39}$	258–288	65–55	FAM	KP331499
	R: *ATTATTCAACATGACACCATTAACC					
PW182	F: *TTGGTGACGACATTAGGAGGC	$(AAAGG)_{30}$	308–318	60–50	FAM	KP331500
	R: TAAGGGCAGCCGACCTAAGC					
PW223	F: *CGTAACACAACCTTACAATCTAAGCC	$(ATC)_{36}$	438–447	65–55	HEX	KP331501
	R: TGCTTTGTGAAGATCAGTGGG					

Note: T_a = range of annealing temperatures used in touchdown PCR.

* Indicates the CAG sequence position (5′-CAGTCGGGCGTCATCA-3′).

[a] Allele size is the range of observed alleles (including the CAG sequence length).

[b] Fluorescent dye used for fragment analysis.

and PW223; and PW182 and PW65. Fluorescent dyes used to label each primer can be found in Table 1. Amplicons were visualized on an ABI 3730xl DNA sequencer (Applied Biosystems, Carlsbad, California, USA) and sized with an internal ROX-labeled size standard (GGF500R; Georgia Genomics Facility, Athens, Georgia, USA). Genotyping results were scored using GeneMarker software (version 2.4; SoftGenetics, State College, Pennsylvania, USA). Alleles were binned using the program MsatAllele (Alberto, 2009) according to the core repeat size of the microsatellite.

Data were checked for errors and null alleles using MICRO-CHECKER (van Oosterhout et al., 2004). No errors were detected. Possible null alleles were detected in the Florida and Illinois populations for PW46, in the Illinois and Japanese populations for PW223 and PW106, and in the Illinois population for PW79. Deviation from Hardy–Weinberg equilibrium (HWE) and linkage disequilibrium (LD) were tested in GENEPOP (version 4.2; Rousset, 2008), both with default parameters and Bonferroni corrections. Genetic diversity measures, including the number of alleles and observed and expected heterozygosities, were estimated in GenAlEx (version 6.501; Peakall and Smouse, 2006, 2012).

Because populations were largely monomorphic for one allele, only eight of the 44 possible tests were subject to both LD and HWE analyses. Overall, seven of the eight loci deviated from HWE, but none of the eight loci pairs were in LD ($P > 0.05$). The number of alleles per locus ranged from two to six and observed heterozygosity ranged from 0.00 to 1.00, although average observed heterozygosity was only 0.03 (Table 2). The breeding system of *P. americana* has never been adequately described. Armesto et al. (1983) speculated that the species was autogamous because of its high fruit set. Caulkins and Wyatt (1990) bagged inflorescences while still in bud and 60% of fruits set, but bagged emasculated flowers (which should not set any fruit) set 46% of their fruit, making their results difficult to interpret. A lack of within-population genetic variation does suggest that *P. americana* may be highly selfing. The allelic differences

TABLE 2. Genetic diversity of 11 newly developed microsatellites in two native (United States) and two invasive (Asia) populations of *Phytolacca americana*.[a]

	Florida, USA (n = 25.9/26)			Illinois, USA (n = 17.7/18)			Anhui, China (n = 24.0/25)			Fukushima, Japan (n = 23.4/24)			All samples (n = 93)
Locus	A	H_o[b]	H_e	A	H_o[b]	H_e	A	H_o[b]	H_e	A	H_o	H_e	A
PW11	1	0.00	0.00	1	0.00	0.00	1	0.00	0.00	1	0.00	0.00	2
PW29	1	0.00	0.00	1	0.00	0.00	1	0.00	0.00	1	0.00	0.00	3
PW46	3	0.12**	0.53	3	0.00*	0.20	1	0.00	0.00	1	0.00	0.00	5
PW53	1	0.00	0.00	1	0.00	0.00	1	0.00	0.00	1	0.00	0.00	2
PW54	2	1.00**	0.50	1	0.00	0.00	1	0.00	0.00	1	0.00	0.00	3
PW65	1	0.00	0.00	1	0.00	0.00	1	0.00	0.00	1	0.00	0.00	2
PW69	1	0.00	0.00	1	0.00	0.00	1	0.00	0.00	2	0.04	0.04	3
PW79	1	0.00	0.00	2	0.12*	0.48	1	0.00	0.00	1	0.00	0.00	2
PW106	1	0.00	0.00	4	0.06**	0.52	4	0.05**	0.25	2	0.05	0.05	6
PW182	1	0.00	0.00	1	0.00	0.00	1	0.00	0.00	1	0.00	0.00	2
PW223	1	0.00	0.00	3	0.00*	0.43	2	0.00	0.08	1	0.00	0.00	3

Note: A = number of alleles; H_e = expected heterozygosity; H_o = observed heterozygosity; n = average number of individuals scored for all loci out of the total number of individuals attempted for a locality.

[a] Locality and voucher information for the populations is available in Appendix 1.

[b] An asterisk (*) indicates that the significance level for a χ^2 test of Hardy–Weinberg equilibrium (HWE) was $P < 0.001$. The double asterisk (**) indicates that the significance level for a χ^2 test of HWE was $P < 0.00001$.

between populations are also consistent with autogamy. Although our markers cannot be used to distinguish between individuals of the same population, they can distinguish between individuals from different populations.

CONCLUSIONS

We report 11 microsatellite loci in *P. americana* from both the native range in the United States and the invasive range in Asia. The genetic diversity data suggest that this species may be highly selfing, although a more detailed examination of the breeding system of this species is warranted. Even though these markers will be of limited use within populations, they should be useful for studies across populations, including those tracing the invasion history of this species. Only one marker was able to amplify any product in the Chinese native congener, *P. acinosa*, suggesting high genetic differentiation between the two species.

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Appendix 1. Voucher information for *Phytolacca* species used in this study.

Species	Voucher specimen accession no.[a]	Collection locality[b]	Geographic coordinates	*N*
Phytolacca americana	FL1-HAD	Williston, Florida, USA	29.462617, −82.450328	26
Phytolacca americana	IL1-KEB	Nashville, Illinois, USA	38.352300, −89.379167	18
Phytolacca americana	PAN2-RM/MMM	Tangkou, Anhui, China	29.927537, 118.026423	25
Phytolacca americana	PJP6-RM	Sukagawa, Fukushima, Japan	37.280853, 140.359136	24
Phytolacca acinosa	CPN-RM	Nanjing, Jiangsu, China	32.060703, 118.834628	24

Note: *N* = number of individuals.

[a] HAD = Hollis A. Dahn, collector; KEB = Kerin E. Bentley, collector; MMM = Margalit M. Mauricio, collector; RM = Rodney Mauricio, collector. Vouchers deposited at the University of Georgia, Department of Genetics, Germplasm bank.

[b] Locality (closest municipality) and state, province, or prefecture.