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PRIMER NOTE

ISOLATION OF 18 MICROSATELLITE LOCI IN THE DESERT MISTLETOE *PHORADENDRON CALIFORNICUM* (SANTALACEAE) VIA 454 PYROSEQUENCING[1]

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- *Premise of the study:* Microsatellite primers were developed for the parasitic mistletoe *Phoradendron californicum* to investigate to what extent population genetic structure depends on host tree distribution within a highly fragmented landscape.
- *Methods and Results:* Fourteen unlinked polymorphic and four monomorphic nuclear microsatellite markers were developed using a genomic shotgun pyrosequencing method. A total of 187 alleles plus four monomorphic loci alleles were found in 98 individuals sampled in three populations from the Sonoran Desert in the Baja California peninsula (Mexico). Loci averaged 13.3 alleles per locus (range 4–28), and observed and expected heterozygosities within populations varied from 0.167–0.879 and 0.364–0.932, respectively.
- *Conclusions:* Levels of polymorphism of the reported markers are adequate for studies of diversity and fragmentation in natural populations of this parasitic plant. Cross-species amplifications in *P. juniperinum* and *P. diguetianum* only showed four markers that could be useful in *P. diguetianum.*

Key words: mistletoe; parasitic plant; *Phoradendron californicum*; shotgun sequencing; SSR; toji.

Mistletoes are parasitic plants dispersed by animals, mainly birds (Watson, 2001). The mistletoe *Phoradendron californicum* Nutt. has a broad distribution in North America and is a native parasitic plant of leguminous plants in the Sonoran Desert, mainly *Prosopis articulata* S. Watson and *Cercidium microphyllum* (Torr.) Rose & I. M. Johnst. The interactions among mistletoes, their host plants, and their animal dispersers are currently under study in both natural and fragmented landscapes in the desert of the Baja California peninsula. Mistletoe–host plants' antagonistic interactions, along with animal dispersers, may form complex networks whose function and structure can be influenced by fragmentation at different scales, e.g., molecular or populations. Thus, the mistletoe-host interaction is ideal for studies on the effects of landscape fragmentation on plant-animal interactions.

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Our aim is to determine to what extent landscape fragmentation affects mistletoe-animal interactions, the dispersal process of seeds, and the resulting networks of spatial genetic variability. For these purposes, we isolated and characterized nuclear microsatellite markers that are being successfully applied to describe spatial patterns of genetic structure. To date, microsatellite primers have not been developed for this mistletoe species.

METHODS AND RESULTS

We extracted genomic DNA using a DNeasy Plant Mini Kit (QIAGEN, Valencia, California, USA) and used ~5 µg from one *P. californicum* individual collected in Santo Domingo Valley, Baja California Sur, Mexico (see Patch 33 population in Appendix 1 for GPS coordinates and voucher), to construct an enriched genomic library digested with *Rsa*I (Sigma-Aldrich Corporation, St. Louis, Missouri, USA) for microsatellite loci using the oligonucleotide probes $(GT)_{15}$, $(CT)_{15}$, $(GATA)_{10}$, $(GACA)_8$, and $(GATGT)_5$ (Glenn and Schable, 2005). We sequenced a total of 288 *Escherichia coli* clones, from which 65 (22.5%) contained microsatellite loci but only 17 (5.9%) had flanking sequences adequate for the design of PCR primers, using Primer3 software (Rozen and Skaletsky, 2000). From these, only one polymorphic locus (Phca63B) was successfully amplified and scored. To overcome the low efficiency of enrichment and cloning methods, we used ~5 µg of genomic DNA from the same individual to construct a shotgun genomic library that was sequenced on 1/8th of a plate using 454 GS FLX Titanium chemistry (Roche Applied Science, Indianapolis, Indiana, USA) at the University of Arizona Genetics Core (Tucson, Arizona, USA). We generated 70.1 Mb of quality-filtered data, distributed over 196,512 unique reads with an average length of 357 bp after quality filtering (quality score [Q] ≥ 20 using a 10-bp sliding window). We located microsatellite

TABLE 1. Characteristics of 18 microsatellite markers isolated from populations of *Phoradendron californicum*.

Locus[a]	Primer sequences (5′–3′)	Repeat motif	Allele size range (bp)	*A*	T_a (°C)	Dye[b]	Mix no. for capillary electrophoresis[c]
PhcaWDS	F: TGATGATGTTACCCAATAGGCA	$(AT)_{10}$	193–219	12	53	FAM	1
	R: CATAATTCACACCTTAGTGGCA						
PhcaIK7	F: AATCCAAACCACTTCATTATCTTT	$(TAT)_8$	137–191	14	53	VIC	1
	R: TGAATGGTGGTGATATTAATCATAAAA						
PhcaK5R	F: GGCCACCATAGGGAATGATA	$(AT)_{12}$	200–235	15	55	VIC	1
	R: TTACCCCATCAATGGTTTCC						
Phca54T	F: CATTCAACTGATGGATTCCAAA	$(ATT)_8$	257–322	18	53	VIC	1
	R: GAAGATCATTAATAGCATGTTCCA						
Phca0Q7	F: GCACTACTATGATTACAATGACAACA	$(CAA)_8$	178–190	4	53	NED	1
	R: TTTTGTAGTGGCAGTAGTTGTGG						
PhcaZ7Y	F: AAAGTGAATGTTGGAGGAATTTTT	$(GAT)_{13}$	166–222	24[d]	55	PET	1
	R: TTTGTGTCGAAGAGTATGTAAATGG						
Phca0GH	F: TAGAACTCCCTCAGACCCCA	$(TTA)_9$	147–212	24[d]	55	FAM	2
	R: TGCATCAATAAGGATGGGATG						
PhcaREF	F: ACATAATCATGGGCCGAAAA	$(AT)_{11}$	258–280	6	56	FAM	2
	R: TGGACATTCCATGGTCATCA						
PhcaCC3	F: CCTTACAGGCCCATCATTCT	$(CAA)_8$	288–308	9[d]	54	FAM	2
	R: TAACAGGTCGCATTGGCATA						
Phca63B	F: CCAAAGGACTTTAAACCTCATTCC	$(GC)_5(CA)_4CG(CA)_{14}(TA)_5$	109–159	12	56	VIC	2
	R: GAGGAAATTATCATCATCCCGTAG						
PhcaUT7	F: TCAAATGTTGCAAAACAAAACA	$(AAT)_8$	276–300	7	54	VIC	2
	R: GGTGTTTTTACATCAATAAACAATGA						
Phca1HP	F: TGATTTTTCGGTTCATGGTT	$(TAA)_{10}$	179–277	28	53	NED	2
	R: GGGTGCCACACATCACAAT						
PhcaGQL	F: TATTGCAATGGGACAAGGCT	$(TTG)_8$	197–246	9	56	PET	2
	R: GTTTCGAACTTGAGACCCCA						
Phca1G8	F: TTCAAGCTTATCCCCACAGC	$(AC)_{12}$	271–279	5	53	PET	2
	R: CCAATACAATAGACCCTAAAAACAA						
PhcaK4A	F: TCTTGTTGTTTCATCGGTGC	$(AT)_{10}$	120	1	55	NED	3
	R: TCAGCAATCCCTCCCATTAC						
PhcaW15	F: TGAGGAGTGTACTCACCCATGT	$(AT)_{12}$	105	1	55	VIC	3
	R: CAACATACCACCACGAGATTG						
PhcaBVY	F: ATCATAAAACCATGCTCGGG	$(AT)_{10}$	129	1	55	PET	3
	R: GAAACTTGATTGTGATGTTCTAAGGA						
PhcaSRK	F: TTCCAGACATGGAGTCGAGA	$(AT)_{10}$	285	1	55	PET	3
	R: AAGACCGGAGCTATCAACCA						

Note: *A* = number of alleles; T_a = annealing temperature (provided for nontailed primers).

[a] Sequences of 17 loci from the shotgun genomic library were submitted to the Sequence Read Archive (SRA) of the National Center for Biotechnology Information (NCBI; SRP015980 study accession). Locus Phca63B has a JX961681 GenBank accession number.

[b] PCR products were labeled using FAM, VIC, NED, or PET (Applied Biosystems) dyes on an additional 19 bp M13 primer (5′-CACGACGTTGTAAAACGAC-3′) according to the methods of Boutin-Ganache et al. (2001). Moreover, a palindromic sequence tail (5′-GTGTCTT-3′) was added to the 5′ end of the reverse primer to improve adenylation and facilitate genotyping.

[c] Mix number indicates loci that were mixed in the same capillary electrophoresis run.

[d] Loci PhcaZ7Y, Phca0GH, and PhcaCC3 showed several alleles (6, 4, and 2, respectively) with a size difference of 2 or 1 bp relative to contiguous alleles; however, no variation outside of the microsatellite region was showed by these loci, and no scoring errors were detected by MICRO-CHECKER software (van Oosterhout et al., 2004). These polymorphisms could result from nonstepwise mutations in the repeat array.

loci containing at least 10 perfect repeats and designed primers using the software QDD (Meglécz et al., 2010). We used unique sequence reads and consensus sequences within contigs that grouped sequences with a similarity ≥95% in regions ≥30 bp flanking the repeats to design primers. This step eliminated duplicated loci that have diverged in the flanking regions (except recent duplicates) and reduced null alleles by assembling sequence data from regions with coverage ≥1× for anchoring primers.

We found 115 di-, 376 tri-, and 13 tetranucleotide loci that met our criteria, and selected all of the tetranucleotides, 24 dinucleotides, and 24 trinucleotides with the largest number of repeats for primer synthesis. For primer testing, DNA was isolated from silica-dried stems using a modified cetyltrimethylammonium bromide (CTAB) extraction method (Milligan, 1998) that included tissue grinding in a Mixer Mill MM301 (Retsch, Haan, Germany) and TLE resuspension (10 mM Tris-HCl [pH 8.0], 0.1 mM EDTA). We sampled a total of 98 *P. californicum* individuals from three natural populations located in the Sonoran Desert in the Baja California peninsula, Mexico (Appendix 1). Sampling was designed to capture at least one sample from each parasitized host tree in each patch. Hosts were more aggregated in patch 32 compared to other patches.

PCR amplifications were performed in a 20-μL final volume containing 1× buffer (67 mM Tris-HCL [pH 8.8], 16 mM $(NH_4)_2SO_4$, 0.01% Tween-20), 2.5 mM $MgCl_2$ (1.5 mM for locus PhcaGQL), 0.01% bovine serum albumin (BSA; Roche Diagnostics, Barcelona, Spain), 0.25 mM dNTP, 0.40 μM dye-labeled M13 primer, 0.40 μM "pig-tailed" reverse primer, 0.04 μM M13-tailed forward primer (see M13 and "pig-tail" sequences in Table 1), 0.5 U *Taq* DNA polymerase (Bioline, London, United Kingdom), and approximately 50–70 ng genomic DNA. Reactions were undertaken in a "touchdown" PCR protocol in a Bio-Rad DNA Engine Peltier Thermal Cycler (Bio-Rad Laboratories, Hercules, California, USA), with an initial 2 min of denaturation at 94°C; 17 cycles at 92°C for 30 s, annealing at 60–44°C for 30 s (1°C decrease in each cycle), and extension at 72°C for 30 s; 25 cycles at 92°C for 30 s, 44°C for 30 s, and 72°C for 30 s; and a final extension of 5 min at 72°C. Amplified fragments were analyzed on an ABI 3130xl Genetic Analyzer (Applied Biosystems, Foster City, California, USA) and sized using GeneMapper 4.0 (Applied Biosystems) and GeneScan 500 LIZ size standard (Applied Biosystems). No multiplexing was attempted at the PCR stage (see mix for capillary electrophoresis in Table 1).

From a total of 61 loci tested from the next-generation library, four were monomorphic, 27 showed complex or nonspecific amplification, six showed

TABLE 2. Results of screening of 14 polymorphic markers in three populations (Patches 32, 33, and 59) of *Phoradendron californicum*.[a]

	Patch 32					Patch 33					Patch 59				
Locus	*n*	*A*	H_o	H_e	HWE	*n*	*A*	H_o	H_e	HWE	*n*	*A*	H_o	H_e	HWE
PhcaWDS	30	8	0.600	0.789	0.0255†	33	9	0.636	0.752	0.0180	33	7	0.727	0.804	0.1850
PhcaIK7	31	9	0.710	0.843	0.0340	33	10	0.848	0.836	0.9373	34	12	0.706	0.810	0.3190
PhcaK5R	31	10	0.677	0.809	0.0437	33	11	0.636	0.778	0.2530	34	12	0.647	0.864	0.0023*†
Phca54T	31	15	0.710	0.915	0.0004*†	33	15	0.879	0.918	0.1333	34	10	0.824	0.864	0.1622
Phca0Q7	30	2	0.267	0.364	0.1578	30	2	0.167	0.440	0.0012*†	33	4	0.212	0.518	0.0001*†
PhcaZ7Y	30	14	0.300	0.883	0.0000*†	33	14	0.485	0.886	0.0000*†	34	18	0.500	0.921	0.0000*†
Phca0GH	31	17	0.774	0.916	0.0706	33	17	0.697	0.902	0.0004*†	34	15	0.500	0.910	0.0000*†
PhcaREF	31	4	0.548	0.668	0.0570	33	6	0.545	0.726	0.0072†	34	4	0.529	0.597	0.0461
PhcaCC3	30	7	0.533	0.733	0.0334†	31	7	0.516	0.754	0.0004*†	33	6	0.545	0.661	0.0837
Phca63B	31	7	0.323	0.540	0.0025*†	33	8	0.576	0.700	0.1299	34	8	0.382	0.744	0.0000*†
PhcaUT7	31	5	0.355	0.583	0.0250†	33	5	0.455	0.577	0.3080	34	5	0.500	0.562	0.3970
Phca1HP	31	17	0.806	0.924	0.0872	33	17	0.636	0.932	0.0014*†	34	18	0.500	0.918	0.0000*†
PhcaGQL	31	7	0.355	0.682	0.0001*†	32	7	0.531	0.798	0.0001*†	34	8	0.618	0.847	0.0250†
Phca1G8	31	5	0.548	0.764	0.0013*†	33	5	0.515	0.745	0.0000*†	34	4	0.353	0.671	0.0002*†

Note: *A* = number of alleles; H_e = expected heterozygosity; H_o = observed heterozygosity; HWE = nominal *P* values for the test of deviations from Hardy–Weinberg equilibrium; *n* = number of individuals successfully genotyped.

[a] See Appendix 1 for GPS coordinates and voucher information.

* Locus showed significant deviation from Hardy–Weinberg equilibrium (Bonferroni-corrected, $P < 0.0036$).

† Locus showed signs for the presence of null alleles.

high frequencies of null alleles, and 11 failed to amplify. Therefore, we obtained a total of 13 polymorphic loci, adding to a total of 14 polymorphic loci among both libraries (Table 1). We observed a total of 187 alleles for our *P. californicum* sample, averaging overall 13.3 alleles per locus (range 4–28; see Table 2 for heterozygosities). The combined probability of individual identity based on the 14 polymorphic loci was estimated at 1.42×10^{-17} with CERVUS (Kalinowski et al., 2007).

In addition, we tested the 14 polymorphic and the four additional monomorphic loci on 12 and eight individuals of two additional species of *Phoradendron*: *P. diguetianum* Tiegh. and *P. juniperinum* A. Gray (Appendix 1). These species were selected because (1) they belong to the same genus; (2) they are also distributed in the Sonoran Desert, and *P. diguetianum* occurs in the same sampling area; and (3) both species are common and, therefore, easy to collect. Sampling was designed to capture a wider range of potential variation among *Phoradendron* species, covering a more extensive geographic area. DNA isolation of *P. diguetianum* samples was performed as described for *P. californicum*, whereas *P. juniperinum* samples were extracted with the DNeasy Plant Mini Kit (QIAGEN). PCR conditions were as described above for *P. californicum* except for labeling PCR products of the 14 polymorphic loci (see Table 3). Three positive and one negative control were run with each PCR to ensure accurate scoring. *Phoradendron diguetianum* showed four loci (Phca0GH, PhcaCC3, Phca63B, and PhcaW15) with ambiguous locus-specific amplification, and the loci PhcaGQL, PhcaK4A, and PhcaSRK were monomorphic; *P. juniperinum* showed no PCR amplifications.

Table 2 summarizes the features of the 14 loci after inspecting their number of alleles, observed and expected heterozygosities (CERVUS 3.0.3; Kalinowski et al., 2007), and testing for deviations from Hardy–Weinberg equilibrium (HWE; Arlequin 3.5.1.3; Excoffier and Lischer, 2010), gametic disequilibrium (GENEPOP 4.1.4; Rousset, 2008), and the presence of null alleles according to the van Oosterhout method (MICRO-CHECKER 2.2.3; van Oosterhout et al., 2004). We used Bonferroni-corrected *P* values to assess the significance of the results obtained. Several loci among populations showed a significant deviation from HWE (Bonferroni-corrected $P < 0.05/14 = 0.0036$) due to a deficit of heterozygote individuals (Table 2). Only loci PhcaZ7Y and Phca1G8 showed this deviation in all three populations, and Phca0Q7, Phca0GH, Phca63B, Phca1HP, and PhcaGQL showed deviations in two populations. Significant gametic disequilibrium ($P < 0.0036$) was detected for two pairs of loci, but patterns were not consistent among populations (only Phca0GH–PhcaCC3 in population 33 and Phca0GH–Phca54T in population 59 were significant). Signs for the presence of null alleles were suggested by the general excess of homozygotes in eight loci for each population (Table 2). Thus PhcaZ7Y, Phca1G8, and PhcaGQL consistently showed null allele frequencies between 0.128–0.320 in all three populations. In contrast, loci Phca0Q7, Phca0GH, Phca63B, Phca1HP, and PhcaCC3 showed evidence of null alleles at frequencies ≤0.2576 in two populations. These results were not unexpected and could stem from the characteristic pattern of aggregation of parasitic plants, imposing limitations to pollen- and seed-mediated gene flow within aggregations and reduced genetic

TABLE 3. Cross-species amplification testing of *Phoradendron californicum* microsatellite markers in *P. juniperinum* and *P. diguetianum*.[a]

	Species	
Marker name[b]	*P. diguetianum*[c]	*P. juniperinum*
Fam PhcaWDS (174–200)	NSA	NA
Pet PhcaIK7 (118–172)	NA	NA
Vic PhcaK5R (181–216)	NA	NA
Ned Phca54T (238–303)	NSA	NA
Fam Phca0Q7 (159–171)	MP (90–200)	NA
Pet PhcaZ7Y (147–203)	NA	NA
Ned Phca0GH (128–193)	Null[d] (116–222)	NA
Pet PhcaREF (239–261)	NSA	NA
Vic PhcaCC3 (269–289)	LSA[e] (143–219)	NA
Vic Phca63B (90–140)	LSA[f] (173–174)	NA
Vic PhcaUT7 (257–281)	NA	NA
Ned Phca1HP (160–258)	MP (92–201)	NA
Vic PhcaGQL (178–227)	Monomorphic (235)	NA
Pet Phca1G8 (252–260)	NA	NA
Ned PhcaK4A (120*)	Monomorphic (120)	NA
Vic PhcaW15 (105*)	LSA[g] (97–109)	NA
Pet PhcaBVY (129*)	NA	NA
Pet PhcaSRK (285*)	Monomorphic (272)	NA[h]

Note: LSA = microsatellite locus-specific amplification; MP = multiple peaks (>2) were observed for each individual; NA = no amplification; NSA = nonspecific amplification was evidenced by the presence of several peaks in all samples with an unscorable pattern; Null = high frequency of null alleles.

[a] PCR products were labeled using FAM, VIC, NED, or PET (Applied Biosystems) dyes on the 5′ end of the forward primer. A sequence tail (5′-GTGTCTT-3′) was added to the 5′ end of the reverse primer.

[b] Values in parentheses represent the size range (in base pairs) in *P. californicum* using the labeled forward primer.

[c] Values in parentheses represent the size range (in base pairs) in *P. diguetianum* using the labeled forward primer.

[d] Five individuals failed to amplify and seven showed a total of six alleles that differed by 1 bp (in a 116–122 bp range).

[e] One individual showed 143/219-bp genotype. The remaining 11 individuals were 152-bp homozygotes.

[f] Six individuals showed 173/174-bp genotype, five of them 173/173, and one of them 174/174. All showed unscorable peaks in an 83–104 bp range.

[g] The number of alleles was five.

[h] Three individuals showed a 272-bp weak peak.

* The size range was obtained using the 19-bp labeled M13 primer.

differentiation among individuals within these clumps. Consistent and significant deviations from HWE and gametic disequilibrium within but not among populations are unlikely to be caused by null alleles or physical proximity between pairs of loci, and could indicate the presence of marked genetic structure within sampled populations (Francois and Durand, 2010).

CONCLUSIONS

Observed levels of polymorphism suggest that the reported markers are adequate for characterizing spatial genetic structure at reduced spatial scales and for studying aggregation patterns in *Phoradendron* host-parasite relationships. Cross-species amplifications were unsuccessful in *P. juniperinum* and ambiguous in *P. diguetianum*, most likely due to high genetic divergence among the studied *Phoradendron* species.

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Appendix 1. Voucher, number of individuals sampled, and location information for *Phoradendron* species in this study. Vouchers are deposited at the University of Arizona Herbarium, Tucson, Arizona (ARIZ), and the herbarium of the Centro de Investigaciones Biológicas del Noroeste, La Paz, Baja California Sur, Mexico (HCIB).

Species	Locality	Population	Latitude (N)	Longitude (W)	No. of individuals	Herbarium ID	Collection date
P. californicum	Baja California Sur	Patch 32	25°12′53.81″	111°42′28.97″	31	HCIB 28108	5 February 2008
P. californicum	Baja California Sur	Patch 33	25°12′48.34″	111°41′52.21″	33	HCIB 28109	5 February 2008
P. californicum	Baja California Sur	Patch 59	25°06′13.67″	111°41′47.93″	34	HCIB 28110	5 February 2008
P. diguetianum	Baja California Sur	Los Planes	23°59′37.38″	110°03′23.03″	4	HCIB 28112	16 July 2012
P. diguetianum	Baja California Sur	Conquista Agraria	24°05′33.72″	110°45′09.36″	2	HCIB 28113	10 July 2012
P. diguetianum	Baja California Sur	Todos Santos	23°27′50.37″	110°14′42.03″	2	HCIB 28114	19 July 2012
P. diguetianum	Baja California Sur	Cancún	24°42′23.44″	111°33′31.03″	1	HCIB 28115	29 July 2012
P. diguetianum	Baja California Sur	San José Comondú	26°03′30.47″	111°48′25.18″	2	HCIB 28116	25 July 2012
P. diguetianum	Baja California Sur	Rancho Cantarranas	24°50′56.00″	111°04′46.00″	1	HCIB 28117	9 June 2012
P. juniperinum	Arizona	Tucson, AZ	32°22′50.61″	110°57′34.57″	8	ARIZ 398223	11 November 2009