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PRIMER NOTE

ISOLATION AND CHARACTERIZATION OF POLYMORPHIC MICROSATELLITE LOCI IN *SPONDIAS RADLKOFERI* (ANACARDIACEAE)[1]

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- *Premise of the study:* Microsatellite markers were developed for *Spondias radlkoferi* to assess the impact of primate seed dispersal on the genetic diversity and structure of this important tree species of Anacardiaceae.
- *Methods and Results:* Fourteen polymorphic loci were isolated from *S. radlkoferi* through 454 GS-FLX Titanium pyrosequencing of genomic DNA. The number of alleles ranged from three to 12. The observed and expected heterozygosities ranged from 0.382 to 1.00 and from 0.353 to 0.733, respectively. The amplification was also successful in *S. mombin* and two genera of Anacardiaceae: *Rhus aromatica* and *Toxicodendron radicans.*
- *Conclusions:* These microsatellite loci will be useful to assess the genetic diversity and population structure of *S. radlkoferi* and related species, and will allow us to investigate the effects of seed dispersal by spider monkeys (*Ateles geoffroyi*) on the genetic structure and diversity of *S. radlkoferi* populations in a fragmented rainforest.

Key words: Anacardiaceae; microsatellites; *Rhus*; *Spondias*; *Toxicodendron.*

The Anacardiaceae is a family of flowering plants with approximately 81 genera and 800 species. Numerous species within this family are economically important due to the production of agricultural food products such as cashews (*Anacardium occidentale* L.; Mitchell and Mori, 1987), mangos (*Mangifera indica* L.; Mukherjee, 1972), pink peppercorns (*Schinus molle* L.; Barkley, 1944), pistachios (*Pistacia vera* L.; Al-Saghir and Porter, 2012), "ciruela mexicana" or "jocote" (*Spondias purpurea* L.), and "jobo" (*S. mombin* L. and *S. radlkoferi* Donn. Sm.; Airy Shaw and Forman, 1967). Other species are poisonous, such as *Toxicodendron radicans* (L.) Kuntze, which contains strong allergens that cause dermatitis to humans and animals (Pell et al., 2011).

Spondias L. consists of 17 species: seven species in the neotropics (Mexico to Brazil) and 10 species in Asia (Miller, 2011; Pell et al., 2011). In Mexico and Central America, *S. mombin*, *S. purpurea*, and *S. radlkoferi* are the most common species, but *S. purpurea* and *S. mombin* are the most consumed and cultivated species (Miller, 2011; Pell et al., 2011). The fruits of *S. radlkoferi* and *S. mombin* are highly consumed by many bird and mammal species. However, the seeds of both species are relatively large (mean ± SD: *S. radlkoferi*, 3.11 ± 0.43 cm; *S. mombin*, 2.10 ± 0.23 cm in length; Benítez-Malvido et al., 2014), which implies that only a few large-sized mammals, such as spider monkeys (*Ateles geoffroyi* Kuhl), are able to swallow and disperse (through endozoochory) the seeds of these tree species (González-Zamora et al., 2009, 2014; Chaves et al., 2011; Benítez-Malvido et al., 2014). Nevertheless, to date it is largely unknown whether and how seed dispersal by spider monkeys affects the genetic structure and diversity of these tree species. Thus, we developed microsatellite markers for *S. radlkoferi* to: (1) test the impact that primate seed dispersal may have on the genetic diversity and structure of this tree species, and (2) help future studies on the genetic diversity and population structure of *S. radlkoferi* and related species.

METHODS AND RESULTS

Genomic DNA of a single individual of *S. radlkoferi* was isolated (voucher deposited at the herbarium of the Instituto de Ecología, A.C., Pátzcuaro, Mexico [IEB], Appendix 1). Previous to the extraction, a prewash step of leaf tissue was made: washing buffer (10 mM Tris-HCl [pH 8.0], 5 mM EDTA, 0.2 M NaCl, 0.45% of 2-mercaptoethanol, and 1% of polyvinylpyrrolidone [PVP]) was added to the ground leaf tissue and mixed for 1 min. After that, the sample was centrifuged for 5 min at maximal spin. DNA was isolated employing the cetyltrimethylammonium bromide (CTAB) protocol (Doyle and Doyle, 1987). Genomic DNA pyrosequencing was then performed on a Roche 454 GS-FLX Titanium sequencer (454 Life Sciences, a Roche Company, Branford, Connecticut, USA) at the GenoSeq Core of the University of California (Los Angeles, California, USA; http://www.genoseq.ucla.edu). The 454 sequencing reads were assembled into contigs with GS De Novo Assembler software (454 Life

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TABLE 1. Characteristics of 14 microsatellite loci isolated from *Spondias radlkoferi.*

Locus	Primer sequences (5′–3′)	Repeat motif	Allele size range (bp)	Dye	T_a (°C)	GenBank accession no.
SPO2	F: CGCTAGTTGTCATTCGCGG R: GCTTAACCTCTGGAAAGTCGC	$(TA)_9$	219–237	VIC	60	KM489124
SPO3	F: GCAGCAGCCATTTGTGAAAC R: CACGTGTTCCCAGTTATGATTTG	$(TTAA)_6$	172–188	PET	60	KJ619735
SPO4	F: ACACCAACGTTTGCGGAG R: TCTAGGTAGACAGCGACAAATC	$(ATCT)_9$	184–212	FAM	60	KJ619736
SPO6	F: TCTATTTGCGTCCAGGTATTTC R: GAATGGGCACGTTCCTTGG	$(AAT)_8$	100–150	NED	60	KM489125
SPO7	F: ACCATTGGAATGCAGGTTACAG R: AGCATCTTTCAAATTCCGCAC	$(ATGT)_7$	200–225	FAM	60	KM489126
SPO8	F: AACCGCTGCATGGCTAATC R: AGTCAAACACAGTGCAGCC	$(CTT)_7$	170–194	VIC	60	KJ619737
SPO9	F: GGCCCGGGACATGAATCC R: TGGCCCATAACAATTGGTCG	$(ATT)_8$	200–222	PET	60	KM489127
SPO10	F: TGCAACTGCATTGTGATCAAG R: GTGCTGTTTCAATCTCTATGAAC	$(ATGT)_7$	190–210	FAM	60	KJ619738
SPO12	F: GGGTGGAGCTTTACGCAAC R: GAGGGTGACATGGATCCGC	$(CTT)_{10}$	184–214	NED	60	KJ619739
SPO14	F: AGGCTGCTTACCAAGAACG R: TGCTCACATCTGCATAATTTGG	$(CT)_{11}$	198–222	VIC	60	KJ619740
SPO15	F: GGTGGTGACTTCAAAGGGC R: AGGTTGCCCATCGTTTAGC	$(AT)_8$	353–421	FAM	58	KJ619741
SPO18	F: AAGAGTATGGTGCACAGAGG R: ATGGTGCAGATTGCAGAGG	$(CT)_9$	319–333	NED	58	KJ619742
SPO21	F: TAGACTTCGCACCAACCTC R: GCCTCTTATCCCTGTTGCG	$(CT)_6$	303–335	VIC	58	KJ619743
SPO22	F: CGCTAGTTGTCATTCGCGG R: GCTTAACCTCTGGAAAGTCGC	$(AT)_9$	186–244	PET	58	KJ619744
SPO26	F: GGCTTGAGCTTTCAGGTGC R: AACCCTCACTTCGGTTTGC	$(CTT)_5$	169–188	NED	58	KJ619745
SPO31	F: TGTTGGCTCTCAACCACAG R: ACTGACTCTCCTGACACCG	$(AAT)_9$	222–240	FAM	58	KJ619746
SPO40	F: ACCATTGGAATGCAGGTTACAG R: AGCATCTTTCAAATTCCGCAC	$(ATGT)_7$	202–240	VIC	58	KJ619747
SPO44	F: TCGTTAGTCGCATAGAATCCC R: TTGTAGCAAGCCATTGCGG	$(CT)_6$	195–209	PET	58	KJ619748

Note: F = forward primer; R = reverse primer; T_a = annealing temperature.

Sciences, a Roche Company) at the GenoSeq Core. A database of approximately 40,000 sequences was obtained and employed for screening of putative microsatellite motifs using MSATCOMMANDER version 0.8.2 (Faircloth, 2008). From these 40,000 sequences, 607 contained dinucleotide repeats, 696 trinucleotide repeats, and 126 tetranucleotide repeats. The primers flanking the repeat motifs were automatically designed with Primer3 software (Rozen and Skaletsky, 2000).

Based on melting temperature, theoretical amplified fragment size, and microsatellite motif, a set of 44 microsatellite sequences were chosen for testing. To evaluate the polymorphisms at each locus, we used genomic DNA

TABLE 2. Genetic diversity of 14 microsatellite loci in *S. radlkoferi* and *S. mombin.*

	S. radlkoferi (N = 37)					*S. mombin* (N = 20)					
Locus	n	A	A_e	H_o	H_e	n	A	A_e	H_o	H_e	Cross amplification[a]
SPO3	37	5	2.503	0.783	0.600	17	7	2.766	0.470	0.638	R/T
SPO4	36	5	2.579	0.777	0.612	16	7	4.000	0.875	0.750	R/T
SPO8	37	6	2.489	0.973	0.598	17	4	2.513	0.882	0.602	R/T
SPO10	36	5	2.308	0.861	0.566	17	2	2.000	1.00	0.500	R/T
SPO12	37	6	2.514	0.729	0.602	16	7	4.741	0.687	0.789	R/T
SPO14	36	7	3.216	0.833	0.689	16	7	4.830	0.875	0.793	R/T
SPO15	33	12	2.959	0.612	0.623	–	–	–	–	–	–
SPO18	35	3	2.963	0.771	0.662	12	5	3.600	1.00	0.722	–/T
SPO21	34	6	1.546	0.382	0.353	13	7	4.225	0.615	0.763	–
SPO22	36	6	3.677	0.750	0.728	17	6	4.379	1.00	0.771	–
SPO26	36	4	2.385	0.861	0.580	–	–	–	–	–	R/–
SPO31	25	5	3.765	1.00	0.733	14	6	3.379	0.642	0.704	R/T
SPO40	35	5	2.421	0.800	0.586	16	7	3.556	1.00	0.718	R/–
SPO44	37	3	1.740	0.540	0.425	15	7	6.618	0.933	0.848	–

Note: A = number of alleles; A_e = effective number of alleles; H_e = expected heterozygosity; H_o = observed heterozygosity; n = number of individuals genotyped; N = number of individuals in the population sampled.

[a] Cross-species amplification in: R = *Rhus aromatica*; T = *Toxicodendron radicans*; – = not amplified.

from 37 individuals (Appendix 1) collected from the Lacandona rainforest (Marqués de Comillas, Chiapas, Mexico; 16°03′N, 90°45′W). Cross-species amplification was carried out in *S. mombin*, *Rhus aromatica* Aiton, and *Toxicodendron radicans*. PCR amplification was performed in a final reaction volume of 5 µL containing 2× Multiplex PCR master mix (QIAGEN, Valencia, California, USA), 0.4 µM of each forward and reverse primer, and approximately 10 ng of DNA template. PCR amplification was performed in an Eppendorf Mastercycler (Eppendorf, Hamburg, Germany) using the following conditions: first denaturing step 94°C, 15 min; 35 cycles of denaturing 94°C, 30 s; primer annealing at 58°C or 60°C (Table 1), 1 min 30 s; extension 72°C, 1 min, and a final extension at 72°C for 10 min. Loci that were successfully amplified were then tested with a fluorescent forward primer. Fragments were electrophoresed in an ABI PRISM 3130 XL Genetic Analyzer (Applied Biosystems, Foster City, California, USA) with the GeneScan 500 LIZ Size Standard included (Applied Biosystems). PeakScanner software version 1.0 (Applied Biosystems) was used for fragment analysis and final sizing.

In total, 14 polymorphic microsatellite loci were selected (Table 1). Monomorphic primers SPO2, SPO6, SPO7, and SPO9 are also listed in Table 1. The microsatellite sequences have been deposited in GenBank. MICRO-CHECKER (van Oosterhout et al., 2004) was employed for testing scoring errors and null alleles. The number of alleles, effective number of alleles, observed heterozygosity, and expected heterozygosity were determined using GenAlEx version 6.5 (Peakall and Smouse, 2006). In *S. radlkoferi*, the number of alleles per locus ranged from three to 12, and the number of effective alleles varied from 1.546 to 3.765 (Table 2). The observed heterozygosity varied from 0.382 to 1.00, and was higher than the expected heterozygosity, which ranged from 0.353 to 0.733 (Table 2). Twelve out of 14 microsatellite loci were also successfully amplified in the related *S. mombin*, nine loci amplified in *Rhus aromatica*, and eight loci amplified in *Toxicodendron radicans* (Table 2).

CONCLUSIONS

Fourteen new microsatellite markers were isolated and proved to be useful to evaluate the genetic diversity of *S. radlkoferi* and *S. mombin*. Cross-amplification of these microsatellite loci in *Rhus* and *Toxicodendron* suggests that they can be useful in studies of other species within Anacardiaceae. These microsatellites will be useful in determining the genetic diversity and structure of *S. radlkoferi*. In particular, we are using these markers to assess the impact that seed dispersal by spider monkeys may have on the genetic diversity of *S. radlkoferi* in continuous and fragmented tropical rainforest. We will determine the genetic identity of seeds and adults to perform parentage analysis and estimate seed dispersal distances in fragmented tropical landscapes of southeastern Mexico. The dispersal analysis will also help us to determine the movement of spider monkeys within and between forest fragments, a poorly assessed ecological process.

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Appendix 1. Voucher information for the species used in this study.

Species	Voucher no.[a]	Collection locality	Geographic coordinates	*n*
Spondias radlkoferi[b]	244065-3-AGZ	Marqués de Comillas, Chiapas, Mexico	16°16′60″N 90°50′19″W	1
	244066-35-AGZ	Marqués de Comillas, Chiapas, Mexico	16°16′54″N 90°50′23″W	37
S. mombin	244061-1-AGZ	Marqués de Comillas, Chiapas, Mexico	16°16′55″N 90°50′16″W	20
Rhus aromatica	244456-8-ITG	Morelia, Mexico	19°38.719′N 101°09.614′W	10
Toxicodendron radicans	244458-2-ITG	Morelia, Mexico	19°40.644′N 101°09.333′W	6

Note: *n* = number of individuals.

[a] Letters at the end of the voucher number identify the collector: AGZ = Arturo González-Zamora; ITG = Ignacio Torres-García. Vouchers are deposited at the IEB Herbarium (Instituto de Ecología, A.C., Pátzcuaro, Mexico).

[b] Individual used for DNA sequencing.