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Source: Applications in Plant Sciences, 3(1)

Published By: Botanical Society of America

URL: https://doi.org/10.3732/apps.1400099

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Applications in Plant Sciences

Applications in Plant Sciences 2015 3(1): 1400099

Primer Note

Development and characterization of microsatellite loci in the mistletoe *Psittacanthus schiedeanus* (Loranthaceae)[1]

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- *Premise of the study:* Microsatellite primers were developed for the parasitic *Psittacanthus schiedeanus*, a common mistletoe species on cloud forest–adapted tree hosts in Mesoamerica, to investigate intraspecific genetic patterns of diversity and genetic structure.
- *Methods and Results:* Using an enriched library, 10 polymorphic microsatellite loci were developed in *P. schiedeanus*. All loci consisted of dinucleotide repeats. Average alleles per locus were 12 (4–17), and a total of 120 alleles were recorded across 39 individuals from four populations in Mexico. Primers were tested in 11 additional species, but only amplified successfully in *P. calyculatus* and *P. angustifolius*.
- *Conclusions:* The polymorphic loci described will be useful in studies of genetic diversity and genetic population differentiation in natural populations of these parasitic plants, and will provide valuable information to understand the importance of host distribution.

Key words: hemiparasite; Loranthaceae; microsatellites; mistletoe; *Psittacanthus schiedeanus*.

Mistletoes are considered the most damaging pathogens to attack commercially important coniferous and hardwood timber stands (Mathiasen et al., 2008). Despite their negative economic impact, mistletoes are ecologically important in forest ecosystems as they provide food, cover, and nesting sites for a variety of birds, mammals, and insects (Watson, 2001). The geographic range of mistletoes is related to the availability of suitable host trees, and the genetic structuring of mistletoe populations is potentially influenced by the distribution of host populations (Norton and Carpenter, 1998). The genus *Psittacanthus* Mart. (c. 119 species; Loranthaceae), an aerial hemiparasite distributed throughout the Neotropics on a wide range of tree hosts, is distinguished by its large and conspicuous red, yellow, or orange flowers, bulky haustorial connections to the host trees, and large fruits with seeds that lack endosperm (Kuijt, 2009). *Psittacanthus schiedeanus* (Cham. & Schlecht.) G. Don is characteristic of the canopy in the cloud forest edges in Mesoamerica and often parasitizes tall trees (López de Buen and Ornelas, 2002). The hermaphroditic, hummingbird-pollinated flowers are self-compatible (Ramírez and Ornelas, 2010), and ripe, lipid-rich, purplish-black fleshy fruits are dispersed by a variety of resident and migratory bird species (López de Buen and Ornelas, 1999, 2001; Ramírez and Ornelas, 2009). The foraging and flocking behavior and local abundance of birds differ widely (López de Buen and Ornelas, 2001), and consequently affect the spatial patterns of mistletoe seed deposition (López de Buen and Ornelas, 1999; López de Buen et al., 2002). At a local scale, mistletoes can develop specificity on particular host trees depending on the heterogeneity of host patches, which may lead to gene flow changes and the eventual formation of mistletoe races (Overton, 1997; Norton and Carpenter, 1998). Cross-infection experiments, which have proven useful to demonstrate host specificity in other mistletoes (Overton, 1997; Lara et al., 2009), have shown local host adaptation of *P. schiedeanus* on *Liquidambar styraciflua* L. (Ramírez and Ornelas, 2012). However, the parasite-host interaction is predominantly on other host species in areas where *L. styraciflua* is not distributed. Thus, geologic- and climate-driven processes implicated in the fragmentation of the Mesoamerican cloud forests and the distribution of potential host species across a geographic range could have influenced the distribution of genetic variation among populations of *P. schiedeanus*.

Our aim is to determine to what extent the historically fragmented distribution of cloud forest in Mesoamerica and the distributions of host species have affected the spatial genetic variability of *P. schiedeanus* and interactions with its hosts, pollinators, and seed dispersers. For these purposes, we isolated and characterized 10 polymorphic nuclear microsatellite loci that are being successfully applied to describe spatial patterns of genetic structure. To date, microsatellite primers have not been developed for this mistletoe species.

[1]Manuscript received 16 October 2014; revision accepted 21 November 2014.

The authors thank Cristina Bárcenas and Antonio Vásquez for laboratory assistance. Permission to conduct fieldwork was granted by the Mexican government (Instituto Nacional de Ecología, Secretaría de Medio Ambiente y Recursos Naturales [INE-SEMARNAT], SGPA/DGGFS/712/1299/12), and Universidad del Valle de Guatemala (UVAL) and Universidade de São Paulo (USP) herbaria collecting permits. This project was funded by research funds from competitive grants (no. 61710, 155686) from the Consejo Nacional de Ciencia y Tecnología (CONACyT) awarded to J.F.O.

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doi:10.3732/apps.1400099

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TABLE 1. Characteristics of 17 microsatellite loci developed in *Psittacanthus schiedeanus*.

Locus	Primer sequences (5′–3′)	Repeat motif	Allele size range (bp)	Fluorescent dye	GenBank accession no.
Psi1	F: GGTGAAATGTGTGAAATATGGA	$(AG)_{18}$	95–131	6FAM	KP027826
	R: GCACATTGTGTCTCTGCTTG				
Psi29	F: CCAGAGTTAGAGATGATCCAG AGTC	$(AG)_{14}$	139–145	VIC	KP027827
	R: TCCATTTGTCCCTTTTAACCA				
Psi6	F: CATCTTGGCTTGAGG GAACT	$(AG)_{22}$	145–199	NED	KP027828
	R: CCCTCTCCCTCTCTCACTCA				
Psi8	F: TGCACT TTCCCTTCTCGATT	$(GA)_{23}$	209–247	PET	KP027829
	R: CTTTCACATCACCGCTTTCA				
Psi25	F: CGGTTAATCAAACCCATTAAG	$(GT)_{19}$	157–227	6FAM	KP027830
	R: AAGCTAAGGACTAAGAAGTGACA				
Psi15	F: AAGAAAGGGAGATTCCAACC	$(AG)_{14}$	78–102	PET	KP027831
	R: TTTTACATAAAGAGGGCTTATAAATG				
Psi2	F: TCGAAGGTGTTGGAGGAAGA	$(AG)_{21}$	88–130	6FAM	KP027832
	R: ACACACATATACACTTGATGCAC				
Psi16	F: TGAATG GGAGGGAAACTT TG	$(AG)_{10}$	168–180	VIC	KP027833
	R: GGGCATCCACATTTTTCATT				
Psi17	F: CAAAGGGAGGTTGCCTACAA	$(AG)_{12}$	196–208	NED	KP027834
	R: ACAGGGACCAACAGACATCC				
Psi19	F: GTGTGTGTGTGTGTGTGCGA	$(GA)_{17}$	145–179	PET	KP027835
	R: CCGGAAACCTTATCACTGCT				
*Psi7	F: TGGGGTTTTGAATTGTAACAAAA	$(GA)_{12}(GT)_{12}(GA)_{13}$	192		KP027836
	R: GAGGCATTATGACCCGAGTG				
*Psi18	F: TCATGCTCCCACTTATGGAA	$(CT)_9$	163		KP027837
	R: TAGAGGGGGCTCAAAGTGTC				
*Psi21	F: GCTACACAGTGCGTTTACGG	$(AC)_9$	107		KP027838
	R: TGCCAAAAATTTGATGCATAG				
*Psi22	F: TCTGCCCAAAAATACATCCT	$(AC)_{10}$	122		KP027839
	R: CTGGATTTCACGATTGTGTTG				
*Psi24	F: CATTGGGATCTGTGATGCTC	$(GT)_8$	114		KP027840
	R: AAGAACTGGGAGGTGGCATT				
*Psi27	F: ACCAGTTTCTCCAAAACCAAG	$(AG)_7$	100		KP027841
	R: CTCTCTATCTCCACTTCAATTC				
*Psi12	F: CACGAGCATCCTCAAATAGCC	$(GT)_{11}$	122		KP027842
	R: TGTGACATCAGGGGCCATAC				

*Loci untested for polymorphism, probably monomorphic.

METHODS AND RESULTS

Microsatellite isolation was performed by the simple sequence repeat (SSR) development company Genetic Marker Services (Brighton, United Kingdom; http://www.geneticmarkerservices.com). We extracted genomic DNA from a single *P. schiedeanus* (PSI) individual collected in Jardín Botánico Francisco Xavier Clavijero, near the city of Xalapa, Veracruz, Mexico (Appendix 1), with the DNeasy Plant Mini Kit (QIAGEN, Valencia, California, USA) to develop an enriched library, and to design and test primer pairs for microsatellite-containing loci. Enrichment involved incubating adapter-ligated restricted DNA with filter-bonded synthetic repeat motifs: $(AG)_{17}$, $(AC)_{17}$, $(AAC)_{10}$, $(CCG)_{10}$, $(CTG)_{10}$, and $(AAT)_{10}$. We detected and sequenced 29 microsatellite-positive *Escherichia coli* clones, of which 27 contained repeat motifs, and 19 of these loci had sufficient flanking regions to design F/R primer pairs using the primer design software Primer3 (Rozen and Skaletsky, 2000). All repeat motifs were perfect dinucleotides.

TABLE 2. Genetic properties of the 10 newly developed polymorphic microsatellites of *Psittacanthus schiedeanus*.[a]

	Jitotol (n = 5)				Motozintla (n = 7)				Rancho Viejo (n = 19)				Xilitla (n = 8)			
Locus	A	H_o	H_e	HWE	A	H_o	H_e	HWE	A	H_o	H_e	HWE	A	H_o	H_e	HWE
Psi1	4	0.600	0.822	0.1597	2	0.00	0.263	0.0771	11	0.631	0.829	0.1824	7	1.00	0.875	0.6202
Psi29	4	0.800	0.777	0.6939	3	0.571	0.648	1.000	5	0.388	0.495	0.0386	1	—	—	—
Psi6	—	—	—	—	3	0.000	0.666	0.0043*	10	0.555	0.792	0.0021*	8	1.00	0.857	0.1551
Psi8	4	0.750	0.821	0.3172	3	0.200	0.511	0.1105	8	0.705	0.798	0.5665	3	0.375	0.675	0.1994
Psi25	4	0.600	0.777	0.6951	6	0.571	0.868	0.0012*	6	0.473	0.605	0.0359	5	0.500	0.725	0.1767
Psi15	6	0.200	0.911	0.0009*	4	0.333	0.651	0.0699	4	0.473	0.613	0.3026	3	0.375	0.425	0.3835
Psi2	4	0.600	0.733	0.1829	4	0.428	0.648	0.2545	10	0.842	0.832	0.0093	5	0.500	0.533	0.5869
Psi16	3	0.600	0.511	1.000	2	0.428	0.362	1.000	5	0.052	0.482	0.0002*	3	0.500	0.425	1.000
Psi17	—	—	—	—	2	0.428	0.362	1.000	1	—	—	—	1	—	—	—
Psi19	4	0.000	0.800	0.0034*	9	0.571	0.912	0.0058	12	0.500	0.892	0.000*	6	0.625	0.816	0.0678

Note: A = number of alleles sampled; H_e = expected heterozygosity; H_o = observed heterozygosity; HWE = P values of the exact test of Hardy–Weinberg equilibrium; n = number of individuals sampled.

[a]All four populations are located in Mexico. See Appendix 1 for geographic coordinates and voucher information.

*Locus showed significant deviations from Hardy–Weinberg equilibrium after Bonferroni correction ($P < 0.005$).

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TABLE 3. Cross-species amplifications of microsatellite primers developed for *Psittacanthus schiedeanus*.

Species	Psi1	Psi29	Psi6	Psi8	Psi25	Psi15	Psi2	Psi16	Psi17	Psi19
Psittacanthus robustus (Mart.) Mart.	–	–	–	–	–	–	–	–	–	–
Psittacanthus acinarius (Mart.) Mart.	–	–	–	–	–	–	–	–	–	–
Psittacanthus cordatus (Hoffmanns. ex Schult. f.) G. Don	–	–	–	–	–	–	–	–	–	–
Psittacanthus sonorae (S. Watson) Kuijt	–	–	–	–	–	–	–	–	–	–
Psittacanthus ramiflorus (Moc. & Sessé ex DC.) G. Don	–	–	–	–	–	–	–	–	–	–
Psittacanthus mayanus Standl. & Steyerm.	–	–	–	–	–	–	–	–	–	–
Psittacanthus macrantherus Eichler	–	–	–	–	–	–	–	–	–	–
Psittacanthus calyculatus (DC.) G. Don	+	~	–	+	+	~	+	+	+	–
Psittacanthus angustifolius Kuijt	+	+	+	+	+	–	+	+	+	+
Psittacanthus auriculatus (Oliv.) Eichler	–	–	–	–	–	–	–	–	–	–
Psittacanthus rhynchanthus (Benth.) Kuijt	–	–	–	–	–	–	–	–	–	–

Note: + = successful amplification; ~ = amplification of multiple bands; – = failed amplification.

Primer pairs were developed to amplify products ranging from 100–250 bp, to help minimize later multiloading overlap ambiguities during sequencer genotyping. The primers were then tested on seven individuals from different populations (Xilitla, Coacoatzintla, Tlalnelhuayocan, Actópan, La Mancha, Motozintla, Jitotol; Appendix 1) using the same touchdown PCR, to maximize specificity. PCR amplifications were performed in a 25-µL final volume containing 7 pmol of each primer, 1.5 mM of $MgCl_2$, 0.2 mM of each dNTP, 1× PCR buffer, 0.8 µg/µL bovine serum albumin (BSA), 0.5 unit *Taq* polymerase (Promega Corporation, Madison, Wisconsin, USA), and 1.5 µL of DNA diluted 20-fold. Touchdown PCR consisted of 32 cycles of denaturation at 95°C for 60 s, annealing temperature step downs every two cycles of 1°C from 64°C to 59°C (12 cycles), then 10 cycles at 58°C and 10 cycles at 57°C for 60 s, elongation at 72°C for 60 s, and a final extension at 72°C for 5 min. Specificity and active polymorphism were checked on a cooled high-resolution agarose gel. The products were run on 4% MetaPhor agarose gels (Lonza, Basel, Switzerland) in TAE at 10°C. Ten microsatellite loci out of 19 showed specific bands and clearly differed in product sizes among the seven individuals used to test polymorphism, and seven loci showed specific bands but are probably monomorphic. Characteristics of microsatellite loci are shown in Table 1.

To determine the number of alleles per locus, observed and expected heterozygosity, significant deviations of Hardy–Weinberg equilibrium (HWE), and linkage disequilibrium, we amplified the 10 microsatellite loci that showed variation in band sizes in 39 individuals from four distantly located populations (Jitotol, Motozintla, Xilitla, Rancho Viejo; Appendix 1). We amplified microsatellite loci with the Multiplex PCR Kit (QIAGEN) using two mixes, each of five fluorescently labeled primers (Applied Biosystems, Foster City, California, USA; Table 1): Mix 1 (Psi1, Psi29, Psi6, Psi8, Psi25) and Mix 2 (Psi15, Psi2, Psi16, Psi17, Psi19). Multiplex PCR amplifications were performed in a 5-µL final volume containing final concentrations of 1× Multiplex PCR Master Mix, 1 mM of additional $MgCl_2$, 0.08 µM of primer mix, and 1–1.5 µL of DNA, with the following cycling conditions: an initial heat activation at 95°C for 15 min, 28 cycles of denaturation at 94°C for 30 s, annealing at 60°C for 80 s, extension at 72°C for 1 min, and a final extension at 60°C for 30 min. PCR products (1 µL) were run on an ABI-PRISM 310 Genetic Analyzer including the GeneScan 600 LIZ Size Standard (Applied Biosystems). Fragment sizing was performed in GeneMapper 3.2 (Applied Biosystems). The locus Psi6 failed to amplify in one population (Jitotol), and others were monomorphic in specific populations (Psi29 in Xilitla, and Psi17 in Jitotol, Xilitla, and Rancho Viejo). The number of different alleles per locus across populations ranged from four to 17. Observed and expected heterozygosity, deviations from HWE, and linkage disequilibrium between pairs of loci were estimated in Arlequin 3.5.1.2 (Excoffier et al., 2005). Significant deviations from expectations under HWE after Bonferroni correction for multiple comparisons were inconsistently found in two loci from Jitotol and Motozintla, and in three loci from Rancho Viejo, probably due to the presence of null alleles. No significant linkage disequilibrium was detected among paired loci comparisons after Bonferroni correction (Table 2).

Cross-species amplifications of microsatellite loci were performed in one to three individuals of each of 11 species of *Psittacanthus*, with the same conditions above. Most primers amplified successfully only in *P. calyculatus* (DC.) G. Don and *P. angustifolius* Kuijt (Table 3).

CONCLUSIONS

The 10 microsatellites described here are the first to be developed for *P. schiedeanus* and the genus *Psittacanthus*. These polymorphic loci will be useful in studies of genetic diversity and genetic population differentiation and will provide valuable information to understand the importance of host distribution and abiotic factors involved in geographic variation and structure of this widespread mistletoe in Mesoamerica. Cross-species amplifications were successful in closely related *P. calyculatus* and *P. angustifolius*, but unsuccessful in most of the studied *Psittacanthus* species, likely due to their high genetic divergence.

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Appendix 1. Voucher, number of individuals sampled, and location information for *Psittacanthus* species in this study.

Species	Locality	Latitude	Longitude	*n*	Voucher no. (Herbarium ID)[a]
P. acinarius	Brazil, Mato Grosso, Cuíaba	−15°35′56″	−56°05′42″	3	G. Ceccantini 3676 (USP)
P. angustifolius	Mexico, Chiapas, Comitán	16°13′46″	−92°08′01″	2	A. Ortíz-Rodríguez s.n. (XAL)
P. angustifolius	Mexico, Oaxaca, Puerto Escondido	15°43′32″	−96°39′48″	1	E. Ruiz-Sánchez 448 (XAL)
P. auriculatus	Mexico, Oaxaca, El Molino	17°46′14″	−97°44′58″	3	A. Ortiz-Rodríguez s.n. (XAL)
P. calyculatus	Mexico, Michoacán, Maravatío	19°54′00″	−100°27′00″	1	E. Ruiz-Sánchez 414 (XAL)
P. calyculatus	Mexico, Michoacán, Morelia	19°60′05″	−101°23′00″	1	A. González s.n. (XAL)
P. calyculatus	Mexico, Tlaxcala, Tlaxcala	19°17′00″	−98°14′00″	1	C. Lara s.n. (XAL)
P. cordatus	Brazil, Mato Grosso, Cuiabá	−15°35′56″	−56°05′42″	3	G. Ceccantini 3671 (USP)
P. macrantherus	Mexico, Sinaloa, El Palmito	23°33′00″	−105°50′00″	1	E. Ruiz-Sánchez 348 (XAL)
P. mayanus	Mexico, Yucatán, Unucmá	21°02′58″	−89°54′38″	1	Nonvouchered
P. mayanus	Mexico, Yucatán, Cuxtal	20°54′37″	−89°37′15″	1	Nonvouchered
P. mayanus	Mexico, Chiapas, Ocozocuautla	16°47′47″	−93°24′30″	1	A. Ortiz-Rodríguez s.n. (XAL)
P. ramiflorus	Mexico, Chiapas, Berriozabal	16°50′21″	−93°18′11″	3	A. Ortiz-Rodríguez s.n. (XAL)
P. rhynchanthus	Guatemala, Patutul	14°22′24″	−91°08′18″	3	J. J. Vega s.n. (UVAL)
P. robustus	Brazil, Minas Gerais, Serra do Cipó	−19°18′26″	−43°52′33″	3	G. Ceccantini 3589 (USP)
P. schiedeanus	Mexico, San Luis Potosí, Xilitla	21°22′39″	−98°59′35″	8	E. Ruiz-Sánchez 281 (XAL)
P. schiedeanus	Mexico, Veracruz, Clavijero	19°30′47″	−96°56′28″	1	M. T. Mejía 2036 (XAL)
P. schiedeanus	Mexico, Veracruz, Rancho Viejo	19°31′11″	−96°58′22″	19	M. T. Mejía 362 (XAL)
P. schiedeanus	Mexico, Veracruz, Coacoatzintla	19°37′41″	−96°52′56″	1	M. T. Mejía 2043 (XAL)
P. schiedeanus	Mexico, Veracruz, Tlalnelhuayocan	19°34′47″	−96°57′38″	1	M. T. Mejía 2041 (XAL)
P. schiedeanus	Mexico, Veracruz, Actópan	19°23′13″	−96°36′56″	1	M. T. Mejía 2049 (XAL)
P. schiedeanus	Mexico, Veracruz, La Mancha	19°20′43″	−96°36′05″	1	M. T. Mejía 2050 (XAL)
P. schiedeanus	Mexico, Chiapas, Motozintla	15°21′21″	−92°14′54″	7	E. Ruiz-Sánchez 261 (XAL)
P. schiedeanus	Mexico, Chiapas, Jitotol	17°01′47″	−92°50′46″	5	E. Ruiz-Sánchez 263 (XAL)
P. sonorae	Mexico, Sonora, Nacapule	27°59′04″	−111°02′40″	1	Nonvouchered
P. sonorae	Mexico, Sonora, Cruz de Piedra	27°57′25″	−110°40′51″	1	Nonvouchered
P. sonorae	Mexico, Sonora, Paraiso La Manga	27°53′43″	−111°06′55″	1	Nonvouchered

[a] IDs reported below refer to accession numbers in the Instituto de Ecología, A.C. (XAL), Universidad del Valle de Guatemala (UVAL), and the Universidade de São Paulo (USP) herbaria.

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