



## **Development and Characterization of Microsatellite Loci in the Mistletoe *Psittacanthus schiedeanus* (Loranthaceae)**

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## PRIMER NOTE

# DEVELOPMENT AND CHARACTERIZATION OF MICROSATELLITE LOCI IN THE MISTLETOE *PSITTACANTHUS SCHIEDEANUS* (LORANTHACEAE)<sup>1</sup>

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- **Premise of the study:** Microsatellite primers were developed for the parasitic *Psittacanthus schiedeana*, 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. schiedeana*. 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 schiedeana*.

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 schiedeana* (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. schiedeana* 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. schiedeana*.

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. schiedeana* 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.

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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<br>R: GCACATTGTGTCTCTGCTTG      | (AG) <sub>18</sub>                                       | 95–131                 | 6FAM            | KP027826              |
| Psi29  | F: CCAGAGTTAGAGATGATCCAG AGTC<br>R: TCCATTTGTCCCTTTTAACCA | (AG) <sub>14</sub>                                       | 139–145                | VIC             | KP027827              |
| Psi6   | F: CATCTTGGCTTGAGG GAACT<br>R: CCCTCTCCCTCTCTCACTCA       | (AG) <sub>22</sub>                                       | 145–199                | NED             | KP027828              |
| Psi8   | F: TGCAC T TCCCTTCTCGATT<br>R: CTTTCACATCACCGCTTTCA       | (GA) <sub>23</sub>                                       | 209–247                | PET             | KP027829              |
| Psi25  | F: CGGTTAATCAAACCCATTAAG<br>R: AAGCTAAGGACTAAGAAGTGACA    | (GT) <sub>19</sub>                                       | 157–227                | 6FAM            | KP027830              |
| Psi15  | F: AAGAAAGGGAGATTCCAAC<br>R: TTTTACATAAAGAGGGCTTATAAATG   | (AG) <sub>14</sub>                                       | 78–102                 | PET             | KP027831              |
| Psi2   | F: TCGAAGGTGTGGAGGAAGA<br>R: ACACACATATACACTTGATGCAC      | (AG) <sub>21</sub>                                       | 88–130                 | 6FAM            | KP027832              |
| Psi16  | F: TGAATG GGAGGAAACTT TG<br>R: GGGCATCCACATTTTTCATT       | (AG) <sub>10</sub>                                       | 168–180                | VIC             | KP027833              |
| Psi17  | F: CAAAGGGAGGTTGCCACAA<br>R: ACAGGGACCAACAGACATCC         | (AG) <sub>12</sub>                                       | 196–208                | NED             | KP027834              |
| Psi19  | F: GTGTGTGTGTGTGTGTGCGA<br>R: CCGGAAACCTTATCACTGCT        | (GA) <sub>17</sub>                                       | 145–179                | PET             | KP027835              |
| *Psi7  | F: TGGGGTTTTGAATTGTAACAAA<br>R: GAGGCATTATGACCCGAGTG      | (GA) <sub>12</sub> (GT) <sub>12</sub> (GA) <sub>13</sub> | 192                    |                 | KP027836              |
| *Psi18 | F: TCATGCTCCCACTTATGGAA<br>R: TAGAGGGGGCTCAAAGTGTC        | (CT) <sub>9</sub>  | 163                    |                 | KP027837              |
| *Psi21 | F: GCTACACAGTGCCTTACCG<br>R: TGCCAAAAATTTGATGCATAG        | (AC) <sub>9</sub>  | 107                    |                 | KP027838              |
| *Psi22 | F: TCTGCCCAAAAATACATCCT<br>R: CTGGATTTACGATTGTGTTG        | (AC) <sub>10</sub>                                       | 122                    |                 | KP027839              |
| *Psi24 | F: CATTGGGATCTGTGATGCTC<br>R: AAGAAGTGGGAGGTGGCATT        | (GT) <sub>8</sub>  | 114                    |                 | KP027840              |
| *Psi27 | F: ACCAGTTTCTCCAACCAAG<br>R: CTCTCTATCTCCACTTCAATTCT      | (AG) <sub>7</sub>  | 100                    |                 | KP027841              |
| *Psi12 | F: CACGAGCATCCTCAATAGCC<br>R: TGTGACATCAGGGGCCATAC        | (GT) <sub>11</sub>                                       | 122                    |                 | KP027842              |

\*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)<sub>17</sub>, (AC)<sub>17</sub>, (AAC)<sub>10</sub>, (CCG)<sub>10</sub>, (CTG)<sub>10</sub>, and (AAT)<sub>10</sub>. 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*.<sup>a</sup>

| Locus | Jitotol (n = 5) |                |                |         | Motozintla (n = 7) |                |                |         | Rancho Viejo (n = 19) |                |                |         | Xilitla (n = 8) |                |                |        |
|-------|-----------------|----------------|----------------|---------|--------------------|----------------|----------------|---------|-----------------------|----------------|----------------|---------|-----------------|----------------|----------------|--------|
|       | A               | H <sub>o</sub> | H <sub>e</sub> | HWE     | A                  | H <sub>o</sub> | H <sub>e</sub> | HWE     | A                     | H <sub>o</sub> | H <sub>e</sub> | HWE     | A               | H <sub>o</sub> | H <sub>e</sub> | 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<sub>e</sub> = expected heterozygosity; H<sub>o</sub> = observed heterozygosity; HWE = P values of the exact test of Hardy–Weinberg equilibrium; n = number of individuals sampled.

<sup>a</sup>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).

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

| Species   | Psi1 | Psi29 | Psi6 | Psi8 | Psi25 | Psi15 | Psi2 | Psi16 | Psi17 | Psi19 |
|---|------|-------|------|------|-------|-------|------|-------|-------|-------|
| <i>Psittacanthus robustus</i> (Mart.) Mart.                     | –    | –     | –    | –    | –     | –     | –    | –     | –     | –     |
| <i>Psittacanthus acinarius</i> (Mart.) Mart.                    | –    | –     | –    | –    | –     | –     | –    | –     | –     | –     |
| <i>Psittacanthus cordatus</i> (Hoffmanns. ex Schult. f.) G. Don | –    | –     | –    | –    | –     | –     | –    | –     | –     | –     |
| <i>Psittacanthus sonorensis</i> (S. Watson) Kuijt               | –    | –     | –    | –    | –     | –     | –    | –     | –     | –     |
| <i>Psittacanthus ramiflorus</i> (Moc. & Sessé ex DC.) G. Don    | –    | –     | –    | –    | –     | –     | –    | –     | –     | –     |
| <i>Psittacanthus mayanus</i> Standl. & Steyerl.                 | –    | –     | –    | –    | –     | –     | –    | –     | –     | –     |
| <i>Psittacanthus macrantherus</i> Eichler                       | –    | –     | –    | –    | –     | –     | –    | –     | –     | –     |
| <i>Psittacanthus calyculatus</i> (DC.) G. Don                   | +    | ~     | –    | +    | +     | ~     | +    | +     | +     | –     |
| <i>Psittacanthus angustifolius</i> Kuijt                        | +    | +     | +    | +    | +     | –     | +    | +     | +     | +     |
| <i>Psittacanthus auriculatus</i> (Oliv.) Eichler                | –    | –     | –    | –    | –     | –     | –    | –     | –     | –     |
| <i>Psittacanthus rhynchanthus</i> (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 multiload overlap ambiguities during sequencer genotyping. The primers were then tested on seven individuals from different populations (Xilitla, Coacoatzintla, Tlalnelhuayocan, Actopan, 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<sub>2</sub>, 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<sub>2</sub>, 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   | <i>n</i> | Voucher no. (Herbarium ID) <sup>a</sup> |
|-------------------------|-------------------------------------|------------|-------------|----------|---|
| <i>P. acinarius</i>     | Brazil, Mato Grosso, Cuiabá         | –15°35'56" | –56°05'42"  | 3        | G. Ceccantini 3676 (USP)                |
| <i>P. angustifolius</i> | Mexico, Chiapas, Comitán            | 16°13'46"  | –92°08'01"  | 2        | A. Ortiz-Rodríguez s.n. (XAL)           |
| <i>P. angustifolius</i> | Mexico, Oaxaca, Puerto Escondido    | 15°43'32"  | –96°39'48"  | 1        | E. Ruiz-Sánchez 448 (XAL)               |
| <i>P. auriculatus</i>   | Mexico, Oaxaca, El Molino           | 17°46'14"  | –97°44'58"  | 3        | A. Ortiz-Rodríguez s.n. (XAL)           |
| <i>P. calyculatus</i>   | Mexico, Michoacán, Maravatío        | 19°54'00"  | –100°27'00" | 1        | E. Ruiz-Sánchez 414 (XAL)               |
| <i>P. calyculatus</i>   | Mexico, Michoacán, Morelia          | 19°60'05"  | –101°23'00" | 1        | A. González s.n. (XAL)                  |
| <i>P. calyculatus</i>   | Mexico, Tlaxcala, Tlaxcala          | 19°17'00"  | –98°14'00"  | 1        | C. Lara s.n. (XAL)                      |
| <i>P. cordatus</i>      | Brazil, Mato Grosso, Cuiabá         | –15°35'56" | –56°05'42"  | 3        | G. Ceccantini 3671 (USP)                |
| <i>P. macrantherus</i>  | Mexico, Sinaloa, El Palmito         | 23°33'00"  | –105°50'00" | 1        | E. Ruiz-Sánchez 348 (XAL)               |
| <i>P. mayanus</i>       | Mexico, Yucatán, Unucmá             | 21°02'58"  | –89°54'38"  | 1        | Nonvouchered                            |
| <i>P. mayanus</i>       | Mexico, Yucatán, Cuxtal             | 20°54'37"  | –89°37'15"  | 1        | Nonvouchered                            |
| <i>P. mayanus</i>       | Mexico, Chiapas, Ocozocuahtla       | 16°47'47"  | –93°24'30"  | 1        | A. Ortiz-Rodríguez s.n. (XAL)           |
| <i>P. ramiflorus</i>    | Mexico, Chiapas, Berriozabal        | 16°50'21"  | –93°18'11"  | 3        | A. Ortiz-Rodríguez s.n. (XAL)           |
| <i>P. rhynchanthus</i>  | Guatemala, Patutul                  | 14°22'24"  | –91°08'18"  | 3        | J. J. Vega s.n. (UVAL)                  |
| <i>P. robustus</i>      | Brazil, Minas Gerais, Serra do Cipó | –19°18'26" | –43°52'33"  | 3        | G. Ceccantini 3589 (USP)                |
| <i>P. schiedeanus</i>   | Mexico, San Luis Potosí, Xilitla    | 21°22'39"  | –98°59'35"  | 8        | E. Ruiz-Sánchez 281 (XAL)               |
| <i>P. schiedeanus</i>   | Mexico, Veracruz, Clavijero         | 19°30'47"  | –96°56'28"  | 1        | M. T. Mejía 2036 (XAL)                  |
| <i>P. schiedeanus</i>   | Mexico, Veracruz, Rancho Viejo      | 19°31'11"  | –96°58'22"  | 19       | M. T. Mejía 362 (XAL)                   |
| <i>P. schiedeanus</i>   | Mexico, Veracruz, Coacoatzintla     | 19°37'41"  | –96°52'56"  | 1        | M. T. Mejía 2043 (XAL)                  |
| <i>P. schiedeanus</i>   | Mexico, Veracruz, Tlalnahuayocan    | 19°34'47"  | –96°57'38"  | 1        | M. T. Mejía 2041 (XAL)                  |
| <i>P. schiedeanus</i>   | Mexico, Veracruz, Actópan           | 19°23'13"  | –96°36'56"  | 1        | M. T. Mejía 2049 (XAL)                  |
| <i>P. schiedeanus</i>   | Mexico, Veracruz, La Mancha         | 19°20'43"  | –96°36'05"  | 1        | M. T. Mejía 2050 (XAL)                  |
| <i>P. schiedeanus</i>   | Mexico, Chiapas, Motozintla         | 15°21'21"  | –92°14'54"  | 7        | E. Ruiz-Sánchez 261 (XAL)               |
| <i>P. schiedeanus</i>   | Mexico, Chiapas, Jitotol            | 17°01'47"  | –92°50'46"  | 5        | E. Ruiz-Sánchez 263 (XAL)               |
| <i>P. sonorae</i>       | Mexico, Sonora, Nacapule            | 27°59'04"  | –111°02'40" | 1        | Nonvouchered                            |
| <i>P. sonorae</i>       | Mexico, Sonora, Cruz de Piedra      | 27°57'25"  | –110°40'51" | 1        | Nonvouchered                            |
| <i>P. sonorae</i>       | Mexico, Sonora, Paraíso La Manga    | 27°53'43"  | –111°06'55" | 1        | Nonvouchered                            |

<sup>a</sup>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.