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Primer Note

# Isolation and characterization of microsatellite loci for *Smilax sieboldii* (Smilacaceae)[1]

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- *Premise of the study:* Polymorphic microsatellite markers were developed for *Smilax sieboldii* (Smilacaceae), a member of the *S. hispida* group with a biogeographic disjunction between eastern Asia and North America, to study the phylogeography and incipient speciation of this species and its close relatives.
- *Methods and Results:* Transcriptome sequencing produced 47,628 unigenes. Seventeen loci were developed from 122 randomly selected primer pairs. Polymorphism and genetic variation were evaluated for 68 accessions representing five populations of *S. sieboldii*. The number of alleles per locus ranged from four to 18; the expected heterozygosity ranged from 0.59 to 0.92. Twelve loci were successfully amplified in five related species: *S. scobinicaulis*, *S. californica*, *S. hispida*, *S. moranensis*, and *S. jalapensis*.
- *Conclusions:* These novel expressed sequence tag–derived microsatellite markers will facilitate further population genetic research of *S. sieboldii* and its close allies of the *S. hispida* group.

**Key words:** eastern Asian and North American disjunction; microsatellite primers; Smilacaceae; *Smilax sieboldii*; transcriptome sequencing.

The *Smilax hispida* group is a well-supported clade including six species in Smilacaceae (Qi et al., 2013) with a disjunct distribution including eastern Asia (*S. sieboldii* Miq. and *S. scobinicaulis* C. H. Wright), western North America (*S. californica* (A. DC.) A. Gray), eastern North America (*S. hispida* Raf.), and Mexico (*S. moranensis* M. Martens & Galeotti and *S. jalapensis* Schltdl.). *Smilax sieboldii* is a typical element of temperate broad-leaved forests that occurs widely in mainland China, Taiwan, Japan, and Korea. Previous studies based on two cpDNA intergenic regions indicated that at least four biogeographic lineages exist, with each lineage containing at least one private haplotype. This phylogeographic structure is considered to be related to the historical fluctuation of climate and sea level (Zhao et al., 2013). However, this study was limited by the lack of nuclear markers. Therefore, polymorphic microsatellite markers will enhance our understanding of population genetic diversity and historical demography (e.g., gene flow, genetic bottlenecks) and will allow for connecting these patterns to geological and environmental changes.

Existing microsatellite markers for *Smilax* species (Xu et al., 2011; Martins et al., 2013) showed limited transferability and polymorphism for the *S. hispida* group due to phylogenetic distance. Therefore, in the current study we aimed to develop more polymorphic and transferable expressed sequence tag–simple sequence repeat (EST-SSR) markers from the transcriptome, which contains abundant ESTs, based on a high-throughput sequencing approach.

## METHODS AND RESULTS

***Transcriptome sequencing*—**Fresh young leaves of one wild accession of *S. sieboldii* were collected at Tianmu Mountain, Zhejiang Province, China (Appendix 1), and frozen in liquid nitrogen. RNA was extracted using TRIzol Reagent (Invitrogen Life Technologies, Carlsbad, California, USA) and treated with DNase (TaKaRa Bio, Shuzo, Kyoto, Japan) following the manufacturer's instructions. A 2 × 150-bp paired-end RNA-Seq library was prepared following the normalized eukaryote transcriptome library preparation protocol of the Beijing Genomics Institute (Shenzhen, China) and sequenced on the Illumina HiSeq 2500 platform (Illumina, San Diego, California, USA). A total of 65,863,062 raw reads were generated and uploaded to the National Center for Biotechnology Information (NCBI) Sequence Read Archive (accession SRP095761). The raw data were filtered using FASTX-TOOLKIT version 0.0.14 (Gordon and Hannon, 2010) by removing adapter sequences and low-quality reads with >5% unknown bases and/or >15% low-quality bases (quality value <20). Remaining reads were assembled into 66,482 transcripts using TRINITY version 2.3.2

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TABLE 1. Characteristics of 17 newly developed microsatellite loci in *Smilax sieboldii*.[a]

| Locus | Primer sequences (5′–3′) | Repeat motif | Allele size range (bp)[b] | Fluorescent dye[c] | GenBank accession no. | Function[d] | Organism | *E*-value |
|---|---|---|---|---|---|---|---|---|
| SS2 | F: ACTGTAGGAGTTGAGCACAGAGG | $(GA)_{17}$ | 60–100 | FAM | KY404961 | Auxin response factor 15 | *Oryza sativa* subsp. *japonica* | 0 |
| | R: AGATTCGGGAAAACAGAGGAAT | | | | | | | |
| SS5 | F: CAACCCAAAACAAAACAAGAGAG | $(AG)_{12}$ | 96–132 | TAMRA | KY404962 | Hydrolase protein 30 | *Arabidopsis thaliana* | 5E-24 |
| | R: GATACACGGGTAACCACCACC | | | | | | | |
| SS19 | F: ACTTTGCCTATTAAGCATCCGTT | $(CT)_{10}$ | 116–154 | ROX | KY404963 | Polygalacturonase inhibitor | *Pyrus communis* | 5E-98 |
| | R: AGTACTGCTTCCTCCACAACAAG | | | | | | | |
| SS20 | F: AACACACGATCTCAAAGAAGAGC | $(GAA)_{15}$ | 89–122 | FAM | KY404964 | Protein FAF-like, chloroplastic | *Arabidopsis thaliana* | 6E-18 |
| | R: CGTCGTCATCTTCTTCTCTGTTT | | | | | | | |
| SS21 | F: GAATCCTTTCGCTTAGGGAAGT | $(CT)_{12}$ | 107–137 | TAMRA | KY404965 | Probable ADP-ribosylation factor GTPase-activating protein AGD14 | *Arabidopsis thaliana* | 2E-15 |
| | R: CACAAAGAATAAAAGAACGCTCG | | | | | | | |
| SS33 | F: AGTAGGATCCCAGCTTTCTTGAG | $(AG)_{11}$ | 141–179 | HEX | KY404966 | Uncharacterized protein At4g08330, chloroplastic | *Arabidopsis thaliana* | 2E-32 |
| | R: CTCTCTCATCCCCAAATGTTTCT | | | | | | | |
| SS43 | F: CAAGTATCCACAACGAAAACCAT | $(GA)_{11}$ | 154–180 | HEX | KY404967 | Oxygen-evolving enhancer protein 2, chloroplastic | *Fritillaria agrestis* | 7E-119 |
| | R: GTGGAGGAAACATGCAGTTGAT | | | | | | | |
| SS74 | F: GACGGCACCAAGAGAAGAAT | $(CTG)_8$ | 181–241 | FAM | KY404968 | — | — | — |
| | R: GTGGATATCATCACCTCGGG | | | | | | | |
| SS95 | F: GTAGAGGCGCTGGGTTCC | $(TGG)_8$ | 135–180 | ROX | KY404969 | Sulfated surface glycoprotein 185 | *Volvox carteri* PE | 3E-06 |
| | R: GCCAAGCTCTGGAAGAACAC | | | | | | | |
| SS100 | F: GATTAGTGAGAGCTTGGCGG | $(GAG)_9$ | 137–170 | TAMRA | KY404970 | Threonine-protein kinase-like protein At5g23170 | *Arabidopsis thaliana* | 2E-64 |
| | R: ATGCACCAACTCCTTCCAAC | | | | | | | |
| SS103 | F: ACCATCTGTCCCAGTTGCAT | $(TGG)_{10}$ | 263–281 | ROX | KY404971 | E3 ubiquitin-protein ligase At1g12760 | *Arabidopsis thaliana* | 8E-24 |
| | R: CTCCCGAGGTTGTCAAAGAG | | | | | | | |
| SS108 | F: AAAGGCCCCCAATTATCATC | $(TGC)_{13}$ | 106–124 | FAM | KY404972 | Formin-like protein 5 | *Oryza sativa* subsp. *japonica* | 5E-29 |
| | R: CGGCTGGAGAAGATGAACTC | | | | | | | |
| SS109 | F: CCGGCAAGTATTGAGGATGT | $(ATC)_{14}$ | 139–175 | HEX | KY404973 | — | — | — |
| | R: GGTGGAAGAGCTCAAAGACG | | | | | | | |
| SS113 | F: CTGATTTCCTTCCTGTTACGTTG | $(CTGT)_6$ | 132–172 | TAMRA | KY404974 | — | — | — |
| | R: CAAATAACCGACTTCAGCTCCTA | | | | | | | |
| SS114 | F: TATTCGTGTAAAGATACGTGGGC | $(GTGTGA)_9$ | 137–167 | ROX | KY404975 | DNA-directed RNA polymerase II subunit 1 | *Arabidopsis thaliana* | 6E-09 |
| | R: TCGGCCATTATTTTAATCACATC | | | | | | | |
| SS120 | F: ATATGCCGTCGAGTATCGTCTT | $(GCAGTA)_4$ | 146–200 | ROX | KY404976 | ABC transporter G family member 14 | *Arabidopsis thaliana* | 0 |
| | R: GAGGAGGTGGTGTACAGGGTAAG | | | | | | | |
| SS122 | F: GACGGACTGACTGATACTTGGAT | $(TAGCAC)_4$ | 125–185 | HEX | KY404977 | Protein PHLOEM PROTEIN 2-LIKE A1 | *Arabidopsis thaliana* | 7E-13 |
| | R: GGAATACTCAAGTTCGCCGTATC | | | | | | | |

[a]An annealing temperature of 58°C was used for all loci.
[b]Size range values based on 68 individuals.
[c]Forward 5′ label.
[d]The unigenes containing microsatellite loci were searched against the SWISS-PROT database (http://www.expasy.ch/sprot/); — = not found.

(Grabherr et al., 2011), which were then clustered into 47,628 unigenes with TGICL version 2.1 (Pertea et al., 2003).

***Microsatellite development*—**Using the MIcroSAtellite identification tool (MISA) (Thiel et al., 2003), microsatellite regions in the unigenes were screened according to the following criteria for repeat numbers: dinucleotide repeats ≥6, trinucleotide repeats ≥5, and tetranucleotide, pentanucleotide, and hexanucleotide repeats ≥4. Primers were designed for the screened microsatellite loci using Primer3 (Untergasser et al., 2012) with the default parameter settings. A total of 9263 microsatellite sequences were obtained, from which 2252 primer pairs were designed. Of these, 122 primer pairs were randomly selected and their forward primers were synthesized with one of three different universal primers (5′-CACGACGTTGTAAAACGAC-3′, 5′-TGTGGAATTGTGAGCGG-3′, or 5′-CTATAGGGCACGCGTGGT-3′) (Boutin-Ganache et al., 2001; Sakaguchi and Ito, 2014). To prevent primer dimers, hairpin structures, and mismatches, the best matches of forward primers and universal primers were selected using OLIGO version 6.67 (Molecular Biology Insights, Cascade, Colorado, USA).

We selected 12 accessions from various populations (Appendix 1) to test the effectiveness of primer amplification and to preliminarily assess genetic variation. Total genomic DNAs were extracted from silica-dried leaves using Plant DNAzol (Invitrogen Life Technologies). PCR amplifications were performed following the standard protocol of the Tsingke PCR kit (Tsingke Biotech Company, Beijing, China) in a final volume of 10 μL, which contained approximately 5 ng of DNA, 5 μL of 2× PCR Master Mix, 0.1 μM of forward primer, 0.4 μM of reverse primer, and 0.3 μM of fluorescently labeled universal primer (FAM, ROX, HEX, TAMRA; Table 1). The PCR thermal profile involved an initial denaturation at 95°C for 5 min; followed by 35 cycles of 94°C for 40 s, 58°C for 30 min, 72°C for 30 s; and a final 10-min extension step at 72°C. Fragment lengths of PCR products were analyzed on a 3730xl DNA Analyzer (Applied Biosystems, Foster City, California, USA) with GeneScan 500 LIZ as an internal reference (Applied Biosystems). Electrophoresis peaks were scored using GeneMarker version 2.2.0 (SoftGenetics, State College, Pennsylvania, USA). A total of 17 primer pairs with stable repeatability and high variation were selected for further analysis. All primer sequences obtained from this study were submitted to GenBank (Table 1).

***Polymorphism assessment*—**To further evaluate the applicability of these primers, 68 individuals from five representative populations from China, Korea, and Japan (Appendix 1) were used to calculate genetic variation parameters. DNA extraction, PCR amplification, and length assessment of PCR products were performed following the procedures described above. The presence of null alleles and their bias on genetic diversity were evaluated based on the expectation maximization method implemented in FreeNA (Chapuis and Estoup, 2007). Deviation from Hardy–Weinberg equilibrium for each population and linkage disequilibrium for each primer pair were tested using GENEPOP version 4.0.7 (Rousset, 2008). The number of alleles, observed heterozygosity, expected heterozygosity, and polymorphism information content were calculated to assess the genetic polymorphism at each locus using CERVUS version 3.0.3 (Kalinowski et al., 2007).

Two loci (SS20, SS95) with high occurrence of null alleles (>5%) were excluded from the following analysis. No significant deviation from Hardy–Weinberg equilibrium ($P < 0.001$) was observed for the remaining 15 loci except SS5 in populations CZJ and JFS; SS19 in population KMJ; and SS21, SS100, and SS109 in population JFS, which might be caused by Wahlund effect of specific populations. There was no evidence of significant linkage disequilibrium in any pair of loci. We detected 156 alleles in total, and the number of alleles at each locus ranged from four to 18, suggesting a moderate to high level of polymorphism. The observed heterozygosity, expected heterozygosity, and polymorphism information content for each locus ranged from 0.36 to 0.97, 0.59 to 0.92, and 0.53 to 0.91, respectively (Table 2).

***Transferability evaluation*—**Transferability of the 15 primers was examined in the accessions of the five related species, i.e., five accessions each for *S. californica*, *S. hispida*, *S. moranensis*, and *S. jalapensis* and 10 accessions for *S. scobinicaulis* (Appendix 1). All loci were successfully amplified except two loci (SS21 and SS100) for *S. hispida* and one (SS33) for *S. moranensis* (Table 3). Polymorphism was detected in all but two loci (SS21 and SS100) for *S. californica*, five (SS2, SS19, SS103, SS120, and SS122) for *S. hispida*, four (SS21, SS74, SS103, and SS114) for *S. moranensis*, and one (SS100) for *S. jalapensis* (Table 3). The levels of both cross-amplifiability and polymorphism largely decreased with increasing phylogenetic distance. In total, 12 loci were amplifiable across the other five species in the *S. hispida* group.

TABLE 2. Genetic properties of the 15 newly developed microsatellite loci for *Smilax sieboldii*. Loci SS20 and SS95 are not included due to a high proportion (>5%) of null alleles.[a]

| | CTW ($n = 6$) | | | | CZJ ($n = 14$) | | | | CJS ($n = 15$) | | | | KMJ ($n = 16$) | | | | JFS ($n = 17$) | | | | Total ($n = 68$) | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Locus | *A* | $H_o$ | $H_e$ | PIC | *A* | $H_o$ | $H_e$ | PIC | *A* | $H_o$ | $H_e$ | PIC | *A* | $H_o$ | $H_e$ | PIC | *A* | $H_o$ | $H_e$ | PIC | *A* | $H_o$ | $H_e$ | PIC |
| SS2 | 4 | 0.83 | 0.77 | 0.65 | 8 | 1.00 | 0.86 | 0.80 | 8 | 0.87 | 0.85 | 0.80 | 9 | 0.88 | 0.86 | 0.82 | 10 | 0.82 | 0.84 | 0.79 | 17 | 0.88 | 0.92 | 0.90 |
| SS5 | 4 | 1.00 | 0.78 | 0.65 | 10 | 0.86 | 0.88 | 0.83* | 7 | 0.93 | 0.83 | 0.78 | 8 | 1.00 | 0.81 | 0.76 | 6 | 0.94 | 0.82 | 0.76* | 14 | 0.94 | 0.90 | 0.88 |
| SS19 | 4 | 0.83 | 0.74 | 0.62 | 7 | 0.79 | 0.74 | 0.67 | 4 | 0.60 | 0.71 | 0.63 | 8 | 0.81 | 0.87 | 0.83* | 7 | 0.65 | 0.79 | 0.74 | 13 | 0.72 | 0.87 | 0.85 |
| SS21 | 2 | 0.67 | 0.49 | 0.35 | 5 | 0.71 | 0.73 | 0.65 | 6 | 0.67 | 0.81 | 0.75 | 1 | 0.00 | 0.00 | 0.00 | 2 | 0.00 | 0.51 | 0.37* | 9 | 0.36 | 0.79 | 0.76 |
| SS33 | 5 | 1.00 | 0.74 | 0.64 | 10 | 0.86 | 0.89 | 0.84 | 4 | 1.00 | 0.76 | 0.68 | 7 | 1.00 | 0.77 | 0.71 | 10 | 1.00 | 0.88 | 0.83 | 18 | 0.97 | 0.92 | 0.91 |
| SS43 | 5 | 1.00 | 0.82 | 0.70 | 5 | 0.71 | 0.77 | 0.71 | 4 | 1.00 | 0.72 | 0.64 | 5 | 1.00 | 0.78 | 0.71 | 8 | 0.71 | 0.70 | 0.66 | 12 | 0.87 | 0.87 | 0.85 |
| SS74 | 8 | 1.00 | 0.91 | 0.81 | 5 | 0.46 | 0.63 | 0.55 | 5 | 0.40 | 0.36 | 0.34 | 4 | 0.56 | 0.60 | 0.50 | 9 | 0.77 | 0.79 | 0.74 | 16 | 0.60 | 0.77 | 0.75 |
| SS100 | 4 | 0.67 | 0.71 | 0.60 | 7 | 0.86 | 0.77 | 0.71 | 6 | 0.73 | 0.73 | 0.67 | 5 | 0.86 | 0.74 | 0.67 | 6 | 0.38 | 0.76 | 0.70* | 10 | 0.69 | 0.82 | 0.79 |
| SS103 | 3 | 0.60 | 0.69 | 0.55 | 4 | 0.46 | 0.64 | 0.54 | 4 | 0.73 | 0.72 | 0.63 | 5 | 0.94 | 0.80 | 0.73 | 4 | 0.50 | 0.56 | 0.48 | 5 | 0.66 | 0.76 | 0.71 |
| SS108 | 4 | 0.83 | 0.76 | 0.64 | 5 | 1.00 | 0.68 | 0.59 | 2 | 0.87 | 0.51 | 0.37 | 4 | 0.94 | 0.60 | 0.50 | 4 | 0.94 | 0.64 | 0.54 | 7 | 0.93 | 0.63 | 0.55 |
| SS109 | 4 | 0.50 | 0.56 | 0.48 | 5 | 0.71 | 0.75 | 0.68 | 3 | 0.67 | 0.67 | 0.58 | 5 | 0.69 | 0.80 | 0.74 | 4 | 0.47 | 0.64 | 0.56* | 9 | 0.62 | 0.84 | 0.82 |
| SS113 | 4 | 1.00 | 0.76 | 0.64 | 4 | 0.71 | 0.55 | 0.45 | 5 | 0.87 | 0.63 | 0.56 | 4 | 0.44 | 0.47 | 0.43 | 3 | 0.53 | 0.42 | 0.34 | 9 | 0.66 | 0.59 | 0.53 |
| SS114 | 3 | 0.67 | 0.55 | 0.45 | 3 | 0.79 | 0.62 | 0.53 | 4 | 0.87 | 0.66 | 0.57 | 3 | 0.31 | 0.46 | 0.40 | 3 | 0.77 | 0.55 | 0.47 | 4 | 0.68 | 0.69 | 0.62 |
| SS120 | 3 | 1.00 | 0.67 | 0.54 | 6 | 0.50 | 0.72 | 0.65 | 2 | 0.40 | 0.41 | 0.32 | 4 | 0.69 | 0.73 | 0.65* | 2 | 0.31 | 0.27 | 0.23 | 8 | 0.52 | 0.67 | 0.64 |
| SS122 | 4 | 0.83 | 0.77 | 0.65 | 3 | 0.33 | 0.45 | 0.37 | 4 | 0.33 | 0.41 | 0.37 | 4 | 0.86 | 0.66 | 0.57 | 4 | 0.67 | 0.65 | 0.57 | 5 | 0.58 | 0.70 | 0.64 |

*Note*: *A* = number of alleles sampled; $H_e$ = expected heterozygosity; $H_o$ = observed heterozygosity; *n* = number of individuals sampled; PIC = polymorphism information content.
[a]Voucher and locality information are provided in Appendix 1.
* Significant deviation from Hardy–Weinberg equilibrium ($P < 0.001$).

TABLE 3. Fragment sizes detected in cross-amplification tests of the 15 newly developed microsatellite markers in the remaining five species of the *Smilax hispida* group.[a]

| Locus | *S. scobinicaulis* (*n* = 10) | *S. californica* (*n* = 5) | *S. hispida* (*n* = 5) | *S. moranensis* (*n* = 5) | *S. jalapensis* (*n* = 5) |
|---|---|---|---|---|---|
| SS2 | 66–80 | 72–84 | 72 | 72–76 | 66–84 |
| SS5 | 98–124 | 114–116 | 114–116 | 114–124 | 114–116 |
| SS19 | 116–150 | 132–136 | 140 | 132–138 | 128–138 |
| SS21 | 125–127 | 125 | — | 131 | 123–129 |
| SS33 | 157–179 | 167–177 | 167–179 | — | 167–179 |
| SS43 | 164–176 | 168–188 | 168–170 | 164–176 | 166–168 |
| SS74 | 184–241 | 193–199 | 202–217 | 196 | 199–214 |
| SS100 | 152–170 | 164 | — | 152–164 | 164 |
| SS103 | 263–278 | 272–278 | 272 | 278 | 257–278 |
| SS108 | 106–118 | 109–118 | 106–118 | 106–109 | 94–118 |
| SS109 | 172–175 | 127–142 | 136–142 | 127–136 | 127–151 |
| SS113 | 132–164 | 160–164 | 160–164 | 156–164 | 132–156 |
| SS114 | 137–155 | 137–149 | 143–149 | 137 | 137–155 |
| SS120 | 146–164 | 170–182 | 158 | 152–170 | 164–182 |
| SS122 | 125–179 | 167–191 | 173 | 173–191 | 167–179 |

*Note*: — = amplification failed.
[a]Voucher and locality information are provided in Appendix 1.

## CONCLUSIONS

Using high-throughput sequencing, we sequenced and assembled the transcriptome of *S. sieboldii* without a reference genome. Fifteen EST-SSR markers were successfully developed to evaluate the genetic structure and demography of *S. sieboldiii*, of which 12 are likely to be useful for all six species of the *S. hispida* group.

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APPENDIX 1. Voucher information for *Smilax* species used in this study.

| Species | Population code | Voucher specimens[a] | Collection locality | Geographic coordinates | *n* |
|---|---|---|---|---|---|
| *Smilax sieboldii* Miq. | CTW | *Xiaoxian Liu, 0812003* | Mt. Zhu, Taiwan, China | 23.31000N, 120.50000E | 6 |
| *Smilax sieboldii* | CZJ | *Yalu Ru, Ru150921001* | Mt. Tianmu, Zhejiang, China | 30.37809N, 119.42061E | 14 |
| *Smilax sieboldii* | CJS | *Yunpeng Zhao, HZU00441* | Mt. Longchi, Jiangsu, China | 31.24818N, 119.74551E | 15 |
| *Smilax sieboldii* | KMJ | *Joongku Lee, GG13* | Myeongjisan, Gyeonggi-do, Korea | 37.93458N, 127.47325E | 16 |
| *Smilax sieboldii* | JFS | *Chengxin Fu & Xinjie Jin, Fu1505092* | Fujiyama, Tokyo, Japan | 35.50281N, 138.76985E | 17 |
| *Smilax scobinicaulis* C. H. Wright | | *Pan Li, LP150444* | Mt. Wuzhi, Hubei, China | 31.08961N, 110.88390E | 10 |
| *Smilax californica* (A. DC.) A. Gray | | *Pan Li, LP150436* | Near Shasta Lake, CA, USA | 40.75954N, 122.03657W | 5 |
| *Smilax hispida* Raf. | | *Yunpeng Zhao, 090834* | Croatan National Forest, NC, USA | 36.20339N, 86.98333W | 5 |
| *Smilax jalapensis* Schltdl. | | *Pan Li, US10041* | Teopisca, Chiapas, Mexico | 16.57310N, 92.50445W | 5 |
| *Smilax moranensis* M. Martens & Galeotti | | *Pan Li, US10031* | Mexico City, Mexico | 19.30541N, 99.30743W | 5 |

*Note*: *n* = number of individuals sampled.
[a]Vouchers were deposited in the Herbarium of Zhejiang University (HZU), Hangzhou, Zhejiang, China.