# Development of Microsatellite Loci in Mediterranean Sarsaparilla (Smilax aspera; Smilacaceae) Using Transcriptome Data

Authors: Qi, Zhe-Chen, Shen, Chao, Han, Yu-Wei, Shen, Wei, Yang, Man, et al.

# Development of microsatellite loci in Mediterranean sarsaparilla (*Smilax aspera*; Smilacaceae) using transcriptome data[1]

Zhe-Chen Qi[2,3], Chao Shen[2,3], Yu-Wei Han[2], Wei Shen[2], Man Yang[2], Jinliang Liu[2], Zong-Suo Liang[2,3,5], Pan Li[4,5], and Cheng-Xin Fu[4]

[2]College of Life Sciences, Zhejiang Sci-Tech University, Hangzhou 310018, People's Republic of China; [3]Zhejiang Province Key Laboratory of Plant Secondary Metabolism and Regulation, Hangzhou 310018, People's Republic of China; and [4]Key Laboratory of Conservation Biology for Endangered Wildlife of the Ministry of Education, and Laboratory of Systematic and Evolutionary Botany and Biodiversity, College of Life Sciences, Zhejiang University, Hangzhou 310058, People's Republic of China

- *Premise of the study:* Although several microsatellite markers of *Smilax aspera* (Smilacaceae) have been reported in a previous study, due to universality issues in cross-population amplification, we have newly developed microsatellite markers for *S. aspera* based on transcriptome data to further investigate gene flow and genetic structure of its circum-Mediterranean, East African, and South Asian populations.
- *Methods and Results:* A total of 4854 simple sequence repeat (SSR) primer pairs were designed from 99,193 contigs acquired from public transcriptome data of *S. bona-nox*. Forty-six microsatellite loci were selected for further genotyping in 12 *S. aspera* populations. The number of alleles varied from three to 28, and 93.5% of the developed microsatellite markers could be cross-amplified in least one of three congeneric *Smilax* species.
- *Conclusions:* The SSR markers developed in this study will facilitate further studies on genetic diversity and phylogeographic patterns of *S. aspera* in intercontinental geographical scales.

   **Key words:** deep lineage divergence; intercontinental disjunction; microsatellites; Smilacaceae; *Smilax aspera*; Tethyan vegetation; transcriptome.

*Smilax aspera* L. (Smilacaceae) is a prickly woody climber with sclerophyllous leaves, small dioecious flowers, and fleshy red berries. This species is widespread throughout the circum-Mediterranean region and has a disjunct distribution into the East African upland evergreen forest and South Asian seasonal forest. With its Tethyan disjunction pattern, *S. aspera* represents an ideal model to test the dynamics and evolutionary history of laurel forests in the Late Tertiary period (Mai, 1995; Chen et al., 2014). A previous phylogeographic study (Chen et al., 2014) detected a deep lineage split between Mediterranean and African-Asian populations of *S. aspera* and a complex biogeographical range evolution history based on cpDNA and ITS sequences. However, these markers could not reveal the recent gene flow by pollen dispersal, and they did not provide detailed insights into intra- and interpopulation gene flow and genetic drift. Therefore, more efficient codominant markers such as microsatellites should be developed to allow further study.

Xu et al. (2011) reported 14 simple sequence repeat (SSR) markers of *S. aspera* developed in Greek and Italian populations using dual-suppression PCR, but three of the published primers were not polymorphic. Also, through subsequent cross-population amplification investigation in eight populations from Africa, Asia, and the Mediterranean, they showed lack of universality. Our testing of these markers showed average amplification efficiency of 48.8%, and 71.4% of the markers had amplification efficiency below 60%. Hence more reliable microsatellite markers are needed. Here, we developed 46 variable microsatellite markers for *S. aspera* based on transcriptome data of *S. bona-nox* L. (Matasci et al., 2014), and further tested their cross-amplification in three congeneric *Smilax* L. species. These additional microsatellite markers will secure enough polymorphic loci and provide powerful information to assess genetic characteristics and lineage divergence in natural populations of *S. aspera*.

## METHODS AND RESULTS

A total of 96 individuals of *S. aspera* from 12 populations (eight individuals per population) and three congeneric species were used in this study (Appendix 1). The populations of *S. aspera* encompass seven in the Mediterranean region,

TABLE 1.   Characteristics of 46 microsatellite loci developed for *Smilax aspera*.

| Locus | Primer sequences (5′–3′) | Repeat motif | Allele size (bp) | $T_a$ (°C) | $A$ | GenBank accession no. |
|---|---|---|---|---|---|---|
| S003 | F: TCCCCATTTCTCCTCACTTG<br>R: GCCACTACAACAACTTAGTGATTTTG | $(TTTTC)_5$ | 100 | 53 | 4 | KY358008 |
| S004 | F: GCCCACTTTCATTGCCTTTA<br>R: AATGTGGGCGTGGTAAAAAG | $(TCA)_8$ | 111 | 53 | 15 | KY358009 |
| S006 | F: AAAGGGGATGAGGAGAAGGA<br>R: AAACCACCATGACTCCTCCA | $(AAG)_7$ | 133 | 59 | 9 | KY358010 |
| S007 | F: CTGCTTCCAGACAGAGGAGG<br>R: ACACTTCTTGGGTTGGCATC | $(TGGTT)_5$ | 139 | 59 | 8 | KY358011 |
| S009 | F: GAGTGAGGAGGGAGGAGCTT<br>R: CCGGAGAACCAGATGAAGAC | $(TC)_{33}$ | 159 | 58 | 23 | KY358012 |
| S016 | F: AGAACTTGAGGGTGTGTGGG<br>R: TTCATGCATACTTTTGCCGA | $(T)_{10}(TC)_6$ | 230 | 58 | 16 | KY358013 |
| S028 | F: TAATCCCTCGCGAAATCAAG<br>R: CCCAAAATCGATCGAGAAAA | $(GATC)_5$ | 120 | 53 | 3 | KY358014 |
| S030 | F: AAGCCAAGCAAACCCATTTA<br>R: CACCCTCTGACTCCGAAGAG | $(GA)_{14}$ | 126 | 59 | 15 | KY358015 |
| S034 | F: CAGGGAGTTGGTCCTCAAAA<br>R: ATGGTTGCAAAGAAACACCC | $(T)_{21}$ | 154 | 59 | 12 | KY358016 |
| S046 | F: CTAAGGCGATATCCTCAGCG<br>R: CAGCCACTTGGTATCCACCT | $(GTGGGC)_5$ | 226 | 59 | 7 | KY358017 |
| S049 | F: AAGGGACATTTTTGTTCCCC<br>R: GCAAGTTAAGCAACACAGTTAAGG | $(TAAA)_6$ | 248 | 59 | 4 | KY358018 |
| S052 | F: AGATCCACAGTTCCACCTGC<br>R: GCGCTTGATGTGCTCAAATA | $(AAACTAT)_{10}$ | 266 | 59 | 8 | KY358019 |
| S053 | F: GATCTGGGTTTCTCGTTGGA<br>R: GGCCATTTGGAAGAGACTGA | $(CTGGGA)_5$ | 269 | 59 | 6 | KY358020 |
| S057 | F: GAGATTTCCAGCAAAACCCA<br>R: AGTTTCTGGGCCCTCTGTCT | $(CGAG)_4$ | 291 | 58 | 5 | KY358021 |
| S060 | F: CCATGGTGGACGACTTTCTT<br>R: GCATGGAAACGCCTATGATT | $(GAT)_6$ | 311 | 59 | 3 | KY358022 |
| S062 | F: CTTGGCAACACCAATCAATG<br>R: TGCACGTGATCACTGGATCT | $(TCCT)_7$ | 326 | 59 | 9 | KY358023 |
| S063 | F: CATTTCGATGAATCGTGTGG<br>R: GTAGGGTTCGGTGCTGATGT | $(CATCT)_5(TC)_{23}$ | 332 | 59 | 21 | KY358024 |
| S066 | F: TCGATTTCCACCCATTCTC<br>R: GCTGAGTACTTGAGGGCGTC | $(CGCCAC)_5$ | 354 | 59 | 10 | KY358025 |
| S072 | F: CAGTGCCTCTTCCTTGCTTC<br>R: TATACCCAGGTCTCCGAACG | $(TGG)_5(GTGGCC)_3$ | 402 | 59 | 16 | KY358026 |
| S081 | F: ATTTCGCCACTACCTTGCAC<br>R: ATCCTTCATTCAATGCCGAG | $(CCCT)_6$ | 103 | 50 | 8 | KY358027 |
| S083 | F: GGACTGGATTCCGTTTTGCT<br>R: AGCCAGGACATTGCCTTTAC | $(CCTCTA)_4$ | 105 | 50 | 4 | KY358028 |
| S085 | F: TGTTGGGTGAGCAAAACAAA<br>R: ACCTTTCTCCCCACTTGCTT | $(T)_{16}$ | 109 | 53 | 16 | KY358029 |
| S086 | F: TAATTGGCTTCGGATTGACC<br>R: GGAATTCGTTCTTCCCCATT | $(AG)_9$ | 112 | 50 | 28 | KY358030 |
| S087 | F: GGACTTGGTCATCAGGTCGT<br>R: TTGTGCAACCAAACTCCAGA | $(TC)_{12}TAGGTC(TCGGA)_3$ | 116 | 55 | 21 | KY358031 |
| S089 | F: CACAAGCTTGATGAGGTCCA<br>R: AAGGACACGGACCATGAAAG | $(TGGTT)_3$ | 125 | 53 | 7 | KY358032 |
| S090 | F: AGCAGCCTTGGGCTTATTTT<br>R: TTCTGTTGTGCGGATATTGG | $(TAAAC)_3$ | 132 | 53 | 5 | KY358033 |
| S093 | F: GAAGGGAGGGAGGAGAAGTG<br>R: CCGTTTAAAGATCCCGTCAA | $(AG)_{12}$ | 135 | 53 | 7 | KY358034 |
| S094 | F: TGCTGGAAGAACAACGACTG<br>R: GTTACCGTTGGTCACCTGCT | $(GCTGTT)_4$ | 143 | 50 | 4 | KY358035 |
| S096 | F: TGGATTCATGTGTTTGGCTG<br>R: AAATCAGGCCTCCTCATTGTAA | $(A)_{22}$ | 145 | 55 | 8 | KY358036 |
| S097 | F: CACCTTCTCCTCCTCTTCCC<br>R: TCATCTCCCCTCTTCTTCCC | $(TTC)_8$ | 148 | 51 | 9 | KY358037 |
| S100 | F: CTGGAGATCTCACCCTCTCG<br>R: CAATGAGACAGTCCGGATCA | $(CCCTCT)_3$ | 155 | 50 | 6 | KY358038 |
| S104 | F: AATTGGGATTTGATGATCGC<br>R: CCAAAAACCCACGAGAGAAA | $(TC)_{17}$ | 168 | 53 | 11 | KY358039 |
| S105 | F: GCTGGTACTTCTTCTTGCCG<br>R: ACTTCGAGAACAGCCTCCAA | $(GGCGGA)_3$ | 168 | 55 | 6 | KY358040 |
| S110 | F: TCACGTGTGAGGTTCTAGCG<br>R: TGGCGTCCCAGTGAGTGT | $(AG)_7AA(AG)_{14}$ | 181 | 59 | 3 | KY358041 |
| S113 | F: ACGTAACTCTCGGTGCCATC<br>R: CGTGTGGAAGGGAGGTAAAA | $(AG)_{11}$ | 185 | 55 | 5 | KY358042 |

TABLE 1. Continued.

| Locus | Primer sequences (5′–3′) | | Repeat motif | Allele size (bp) | $T_a$ (°C) | $A$ | GenBank accession no. |
|---|---|---|---|---|---|---|---|
| S116 | F: | ATGACATCCCCTCCCTCTCT | $(TC)_9$ | 191 | 55 | 15 | KY358043 |
| | R: | CCCCACCATTGTCTTGAAGT | | | | | |
| S120 | F: | AGGCCAAGACTATCAGCGAA | $(GTG)_7$ | 204 | 53 | 3 | KY358045 |
| | R: | TCTTTCTTGCTCCAGGCATT | | | | | |
| S121 | F: | GGGAACACTACCTTCTGCCA | $(CGATCT)_4$ | 211 | 61 | 3 | KY358046 |
| | R: | TTGAGATCTGGGGAGGTTTG | | | | | |
| S122 | F: | TGTGGTGCTTGATGAGCTTC | $(CTG)_7$ | 214 | 50 | 3 | KY358047 |
| | R: | CGTTGCACAGAGCGAATAAA | | | | | |
| S126 | F: | CTTCTCCGCATACCACCTGT | $(CT)_{10}$ | 227 | 53 | 6 | KY358048 |
| | R: | GCTCTGCGTCTGTTCCATTT | | | | | |
| S130 | F: | ATGCTTGACACGCTTGATTG | $(TGC)_8$ | 247 | 53 | 12 | KY358049 |
| | R: | AGCTGCTTGGACAGCAAAAT | | | | | |
| S132 | F: | ACGGTCTCTTTCAAGAAGGG | $(AG)_{12}$ | 251 | 55 | 11 | KY358050 |
| | R: | GATGAAGGAGAACGCAAAGC | | | | | |
| S134 | F: | GAGAGCCCACGTGAAGTGAT | $(GA)_{15}$ | 258 | 55 | 27 | KY358051 |
| | R: | CCCCATAAATGTGGGAGATG | | | | | |
| S139 | F: | GCAAAGCTCTTCTCCTCCCT | $(TTC)_5$ | 282 | 50 | 7 | KY358052 |
| | R: | CTGGATGGCTTTGGATAGGA | | | | | |
| S144 | F: | GACCCCATGGATACGAGAAC | $(GGGGTC)_3$ | 306 | 55 | 4 | KY358053 |
| | R: | CTAAACCCGACTCCCCAAAT | | | | | |
| S148 | F: | AGAACCAGCAGAGCGACATT | $(CAG)_7$ | 350 | 55 | 4 | KY358054 |
| | R: | TTGCGTCAGCTTACCCTTCT | | | | | |

*Note*: $A$ = number of alleles per locus; $T_a$ = optimized annealing temperature.

four in South Asia, and one in East Africa. Fresh leaves were collected from each individual and dried in silica gel. Total genomic DNA was extracted following a modified cetyltrimethylammonium bromide (CTAB) protocol (Narzary et al., 2015), which was aided by using a more efficient Plant DNAzol Kit (Thermo Fisher Scientific, Waltham, Massachusetts, USA). Then, DNA quality was examined on 1% agarose gel, and concentration was checked using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific).

In this study, we obtained the transcriptome of *S. bona-nox*, a congeneric species of *S. aspera*, as a source for batch primer design. The raw data were acquired from the National Center for Biotechnology Information (NCBI; accession no. ERR364398) and assembled by Geneious 9.0.2 software (Kearse et al., 2012). In total, 99,193 contigs were prepared for SSR targeting and primer design. Microsatellite (SSR) repeats in contigs were observed by MISA software (Thiel et al., 2003). The SSR search was performed for mono-, di-, tri-, tetra-, penta-, and hexanucleotide repeats with a minimum of 10, six, five, four, three, and three repeats, respectively. The maximum number of bases interrupting two SSRs in a compound microsatellite was 100 bp. Primer pairs were then designed using Primer3 software (Rozen and Skaletsky, 1999). The primer annealing temperature was set from 50°C to 65°C, primer size was between 18 and 27 bp with an optimal size of 20 bp, the product size was from 100 to 500 bp, and the other settings were left at default values. A total of 4854 SSR primer pairs were designed, and 153 pairs were selected randomly based on the proportion of different microsatellite repeats. A cost-effective fluorescent labeling method was applied following Schuelke (2000), and the protocol was optimized according to Sakaguchi and Ito (2014). For all loci, a forward primer was synthesized with an M13 sequence (5′-CACGACGTTGTAAAACGAC-3′) at the 5′ end, and a universal M13 primer (5′-CACGACGTTGTAAAACGAC-3′) labeled with one of four fluorophores (FAM, TAMRA, HEX, ROX) was added during PCR amplification.

The primer pairs were initially tested for successful PCR amplification in 12 individuals from 12 separate populations. PCR amplifications were performed on a T100 Thermal Cycler (Applied Biosystems, Life Technologies, Waltham, Massachusetts, USA) with a 10-µL reaction mixture that contained 1 µL of genomic DNA, 5 µL 2× Master Mix (TSINGKE, Hangzhou, Zhejiang, China), 0.2 µM of forward primers, and 0.2 µM of reverse primers. The PCR protocol used was as follows: an initial denaturation at 94°C for 5 min; followed by 35 cycles at 94°C for 45 s, a temperature gradient from 50°C to 65°C was applied for annealing for 45 s, and 72°C for 1 min; and a final extension at 72°C for 5 min. Amplification products were checked on 2% agarose gel stained with GeneGreen Nucleic Acid dye (TIANGEN, Beijing, China).

Fifty-three primer pairs generated specific amplification products and were used for amplification in 96 individuals from 12 populations, using the two-step PCR protocol described in Schuelke (2000). In the first step, the PCR reaction mixtures were in a final volume of 10 µL, which contained 1 µL of genomic DNA, 5 µL 2× Master Mix, 0.1 µM of forward primers, and 0.4 µM of reverse primers. The PCR conditions involved denaturation at 94°C for 5 min; followed by 35 cycles at 94°C for 45 s, at a locus-specific annealing temperature (Table 1) for 45 s, and 72°C for 1 min; and a final extension at 72°C for 5 min. In the second step, the reaction mixtures contained the same PCR products as in the first step, plus 5 µL 2× Master Mix and another 0.8 µL (5 µM) of fluorophore-labeled universal M13 primer for a final volume of 20 µL. The PCR conditions involved denaturation at 94°C for 3 min; followed by 20 cycles at 94°C for 30 s, annealing at 53°C for 30 s, and 72°C for 45 s; and a final extension at 72°C for 10 min. Then, 1 µL of the fluorescent PCR product was added to 8.8 µL of formamide and 0.2 µL of GeneScan 500 LIZ Size Standard (Applied Biosystems, Life Technologies). Reaction products were subsequently run on an ABI PRISM 3730xl Genetic Analyzer (Applied Biosystems). Genotypes were scored by Geneious version 9.0.2 software (Kearse et al., 2012). Finally, 46 of 53 primer pairs with clear and robust genotype information and suitable genetic variation were selected for further population genetic study. All of the selected loci can be stably amplified in 96 tested individuals (12 populations), except one (locus S062) that could not be amplified in population KL, which makes the amplification efficiency of these primers 97.8%. Information and GenBank accession numbers for the 46 microsatellites are provided in Table 1.

Genetic diversity parameters were estimated using CERVUS 3.0 (Kalinowski et al., 2007), including the number of alleles, observed and expected heterozygosity, and polymorphism information content (Table 2). Deviations from Hardy–Weinberg equilibrium were tested through GENEPOP 4.2 (Rousset, 2008) (Table 2). All parameters were calculated for three groups of *S. aspera* (Mediterranean, East African, and South Asian; Table 2). The polymorphism information content ranged from zero to 0.918, the number of alleles ranged from one to 25, and the expected heterozygosity and observed heterozygosity varied from 0.000 to 0.932 and 0.000 to 1.000, respectively. Also, 10 loci showed significant deviation from expectations under Hardy–Weinberg equilibrium because of an excess of homozygotes. Wahlund effect, inbreeding, null alleles, and sampling effect are all potential causes of the deviation.

To test the congeneric transferability of the 46 selected markers, cross-amplification was performed in three congeneric species (*S. riparia* A. DC., *S. china* L., *S. hugeri* (Small) J. B. Norton ex Pennell; Appendix 1), with five individuals per species. Primer transferability was detected using 2% agarose gels, and amplification was considered successful when one clear distinct band was visible in the expected size range. In total, 93.5% of the developed microsatellite markers could be cross-amplified in at least one of three congeneric *Smilax* species. Specifically, the transferability values in each species were 87.0% in *S. riparia*, 78.3% in *S. china*, and 76.1% in *S. hugeri* (Table 3).

TABLE 2. The genetic parameters (per locus) in three continental groups of *Smilax aspera*.[a]

| Locus | Mediterranean group[b] (N = 56) | | | | East African group[c] (N = 8) | | | | South Asian group[d] (N = 32) | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | $A$ | $H_o$ | $H_e$ | PIC[e] | $A$ | $H_o$ | $H_e$ | PIC[e] | $A$ | $H_o$ | $H_e$ | PIC[e] |
| S003 | 4 | 0.839 | 0.609 | 0.539*** | 2 | 0.625 | 0.458 | 0.337 | 3 | 0.813 | 0.502 | 0.387* |
| S004 | 8 | 1.000 | 0.769 | 0.725* | 4 | 1.000 | 0.675 | 0.570 | 10 | 1.000 | 0.847 | 0.812 |
| S006 | 6 | 0.933 | 0.605 | 0.517*** | 5 | 0.800 | 0.822 | 0.701 | 4 | 1.000 | 0.540 | 0.421*** |
| S007 | 7 | 0.982 | 0.599 | 0.515*** | 3 | 1.000 | 0.592 | 0.456* | 5 | 1.000 | 0.676 | 0.618*** |
| S009 | 20 | 0.648 | 0.909 | 0.892 | 5 | 0.250 | 0.800 | 0.712*** | 12 | 0.719 | 0.799 | 0.763 |
| S016 | 15 | 0.455 | 0.847 | 0.818 | 2 | 0.500 | 0.500 | 0.305 | 3 | 0.100 | 0.099 | 0.094 |
| S028 | 3 | 0.821 | 0.557 | 0.480*** | 2 | 1.000 | 0.533 | 0.375* | 2 | 0.906 | 0.503 | 0.373*** |
| S030 | 15 | 0.714 | 0.896 | 0.879 | 6 | 0.750 | 0.800 | 0.712 | 12 | 0.844 | 0.897 | 0.872 |
| S034 | 5 | 0.964 | 0.662 | 0.592** | 3 | 1.000 | 0.667 | 0.555 | 9 | 1.000 | 0.841 | 0.806 |
| S046 | 6 | 0.714 | 0.561 | 0.513* | 2 | 0.143 | 0.143 | 0.124 | 6 | 0.839 | 0.755 | 0.703 |
| S049 | 3 | 0.682 | 0.498 | 0.382 | 3 | 1.000 | 0.644 | 0.492 | 3 | 0.423 | 0.429 | 0.347 |
| S052 | 7 | 0.804 | 0.649 | 0.601 | 3 | 1.000 | 0.604 | 0.465 | 5 | 0.906 | 0.697 | 0.632 |
| S053 | 2 | 0.196 | 0.179 | 0.161 | 3 | 0.500 | 0.542 | 0.428 | 6 | 0.533 | 0.727 | 0.683 |
| S057 | 5 | 0.537 | 0.491 | 0.456 | 1 | 0.000 | 0.000 | 0.000 | 3 | 0.688 | 0.494 | 0.414 |
| S060 | 3 | 0.327 | 0.375 | 0.335 | 1 | 0.000 | 0.000 | 0.000 | 1 | 0.000 | 0.000 | 0.000 |
| S062 | 7 | 0.564 | 0.648 | 0.582 | NA | NA | NA | NA | 2 | 0.036 | 0.036 | 0.034 |
| S063 | 19 | 0.893 | 0.899 | 0.882 | 4 | 0.667 | 0.712 | 0.599 | 11 | 0.862 | 0.877 | 0.846 |
| S066 | 10 | 0.905 | 0.852 | 0.823 | 3 | 1.000 | 0.733 | 0.535 | 8 | 0.933 | 0.848 | 0.798 |
| S072 | 15 | 0.939 | 0.853 | 0.828 | 6 | 0.857 | 0.857 | 0.766 | 11 | 0.889 | 0.859 | 0.818 |
| S081 | 7 | 0.661 | 0.610 | 0.530 | 3 | 1.000 | 0.633 | 0.511 | 6 | 0.906 | 0.687 | 0.621 |
| S083 | 4 | 0.500 | 0.404 | 0.358 | 3 | 0.250 | 0.242 | 0.215 | 2 | 0.281 | 0.246 | 0.212 |
| S085 | 13 | 0.735 | 0.619 | 0.578 | 3 | 0.500 | 0.591 | 0.460 | 9 | 0.875 | 0.708 | 0.659 |
| S086 | 25 | 0.982 | 0.915 | 0.901 | 7 | 0.625 | 0.742 | 0.666 | 18 | 0.906 | 0.893 | 0.869 |
| S087 | 15 | 0.820 | 0.916 | 0.900 | 4 | 0.714 | 0.780 | 0.674 | 10 | 0.556 | 0.830 | 0.792 |
| S089 | 6 | 0.464 | 0.397 | 0.374 | 3 | 0.250 | 0.242 | 0.215 | 4 | 0.781 | 0.559 | 0.490 |
| S090 | 5 | 0.714 | 0.533 | 0.480** | 4 | 0.625 | 0.517 | 0.443 | 4 | 0.844 | 0.592 | 0.525** |
| S093 | 3 | 0.000 | 0.459 | 0.403 | 2 | 0.000 | 0.667 | 0.375 | 3 | 0.174 | 0.305 | 0.273 |
| S094 | 4 | 0.491 | 0.490 | 0.384 | 2 | 0.500 | 0.429 | 0.305 | 2 | 0.406 | 0.329 | 0.271 |
| S096 | 6 | 0.300 | 0.437 | 0.410 | 2 | 0.286 | 0.264 | 0.215 | 4 | 0.750 | 0.499 | 0.398 |
| S097 | 9 | 0.536 | 0.444 | 0.414 | 4 | 0.625 | 0.517 | 0.443 | 4 | 0.750 | 0.554 | 0.493 |
| S100 | 5 | 0.446 | 0.766 | 0.720 | 1 | 0.000 | 0.000 | 0.000 | 4 | 0.563 | 0.665 | 0.573 |
| S104 | 6 | 0.536 | 0.767 | 0.726 | 3 | 0.125 | 0.542 | 0.428 | 9 | 0.281 | 0.754 | 0.705 |
| S105 | 5 | 0.702 | 0.531 | 0.480* | 1 | 0.000 | 0.000 | 0.000 | 4 | 0.938 | 0.669 | 0.588 |
| S110 | 4 | 0.704 | 0.509 | 0.403 | 3 | 0.625 | 0.492 | 0.398 | 2 | 0.548 | 0.432 | 0.335 |
| S113 | 10 | 0.596 | 0.569 | 0.536 | 4 | 0.667 | 0.800 | 0.620 | 9 | 0.458 | 0.796 | 0.748 |
| S116 | 5 | 0.564 | 0.501 | 0.440 | 2 | 0.625 | 0.458 | 0.337 | 6 | 0.710 | 0.582 | 0.502 |
| S120 | 3 | 0.434 | 0.453 | 0.356 | 2 | 0.429 | 0.363 | 0.280 | 2 | 0.633 | 0.481 | 0.361 |
| S121 | 2 | 0.600 | 0.470 | 0.357 | 2 | 0.714 | 0.538 | 0.375 | 3 | 0.692 | 0.495 | 0.411 |
| S122 | 3 | 0.393 | 0.449 | 0.387 | 1 | 0.000 | 0.000 | 0.000 | 1 | 0.000 | 0.000 | 0.000 |
| S126 | 6 | 0.510 | 0.426 | 0.368 | 2 | 0.250 | 0.233 | 0.195 | 4 | 0.688 | 0.489 | 0.393 |
| S130 | 6 | 0.558 | 0.524 | 0.442 | 3 | 0.750 | 0.667 | 0.555 | 10 | 0.742 | 0.811 | 0.772 |
| S132 | 5 | 0.370 | 0.393 | 0.366 | 3 | 1.000 | 0.621 | 0.477 | 9 | 0.906 | 0.872 | 0.841 |
| S134 | 22 | 1.000 | 0.932 | 0.918 | 4 | 1.000 | 0.692 | 0.592 | 14 | 1.000 | 0.852 | 0.822 |
| S139 | 6 | 0.538 | 0.643 | 0.588 | 2 | 0.333 | 0.600 | 0.375 | 5 | 0.333 | 0.460 | 0.423 |
| S144 | 1 | 0.000 | 0.000 | 0.000 | 1 | 0.000 | 0.000 | 0.000 | 4 | 0.226 | 0.546 | 0.483 |
| S148 | 4 | 0.059 | 0.303 | 0.270 | 1 | 0.000 | 0.000 | 0.000 | 3 | 0.250 | 0.458 | 0.362 |
| Mean | 7.61 | 0.612 | 0.585 | 0.534 | 2.89 | 0.533 | 0.482 | 0.375 | 5.89 | 0.645 | 0.587 | 0.537 |

*Note*: $A$ = number of alleles per locus; $H_e$ = expected heterozygosity; $H_o$ = observed heterozygosity; $N$ = number of individuals sampled; NA = unsuccessful amplification; PIC = polymorphism information content.

[a] Locality and voucher information are available in Appendix 1.

[b] The Mediterranean Group consists of populations PL, SM, IR, IS, GA, GC, and TT.

[c] The East African Group consists of population KL.

[d] The South Asian Group consists of populations SRL, NS, CP, and CJ.

[e] Significant deviations from Hardy–Weinberg equilibrium at $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$, respectively.

## CONCLUSIONS

Forty-six highly polymorphic microsatellite markers were developed successfully in this study and can be applied to elucidate the population structure and possible intra- and interpopulation gene flow of *S. aspera*. The cross-amplification of these SSR primer pairs in three *Smilax* species was successful, which suggests the potential of these markers to clarify underlying genetic introgression as well as cryptic speciation events of *Smilax* species.

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TABLE 3. Cross-amplification efficiency of *Smilax aspera* in three congeneric species.[a]

| Locus | *Smilax riparia* (N = 5) | *Smilax china* (N = 5) | *Smilax hugeri* (N = 5) |
|---|---|---|---|
| S003 | 80.0% | 100.0% | 100.0% |
| S004 | 40.0% | 100.0% | 100.0% |
| S006 | 100.0% | 100.0% | 100.0% |
| S007 | 100.0% | 100.0% | 100.0% |
| S009 | 100.0% | 100.0% | 40.0% |
| S016 | 100.0% | 0.0% | 0.0% |
| S028 | 100.0% | 80.0% | 100.0% |
| S030 | 60.0% | 0.0% | 0.0% |
| S034 | 100.0% | 100.0% | 100.0% |
| S046 | 100.0% | 80.0% | 100.0% |
| S049 | 100.0% | 100.0% | 100.0% |
| S052 | 100.0% | 0.0% | 40.0% |
| S053 | 100.0% | 100.0% | 100.0% |
| S057 | 100.0% | 40.0% | 40.0% |
| S060 | 80.0% | 100.0% | 0.0% |
| S062 | 100.0% | 0.0% | 0.0% |
| S063 | 100.0% | 100.0% | 100.0% |
| S066 | 100.0% | 100.0% | 100.0% |
| S072 | 100.0% | 100.0% | 100.0% |
| S081 | 100.0% | 100.0% | 100.0% |
| S083 | 100.0% | 100.0% | 100.0% |
| S085 | 0.0% | 0.0% | 60.0% |
| S086 | 100.0% | 100.0% | 100.0% |
| S087 | 100.0% | 0.0% | 80.0% |
| S089 | 100.0% | 100.0% | 100.0% |
| S090 | 0.0% | 100.0% | 0.0% |
| S093 | 100.0% | 100.0% | 60.0% |
| S094 | 100.0% | 100.0% | 80.0% |
| S104 | 0.0% | 0.0% | 0.0% |
| S096 | 100.0% | 100.0% | 80.0% |
| S097 | 0.0% | 0.0% | 0.0% |
| S100 | 100.0% | 100.0% | 100.0% |
| S105 | 100.0% | 100.0% | 100.0% |
| S110 | 0.0% | 100.0% | 40.0% |
| S113 | 100.0% | 100.0% | 100.0% |
| S116 | 100.0% | 100.0% | 0.0% |
| S120 | 100.0% | 100.0% | 100.0% |
| S121 | 100.0% | 0.0% | 0.0% |
| S122 | 100.0% | 100.0% | 100.0% |
| S126 | 0.0% | 0.0% | 0.0% |
| S130 | 100.0% | 100.0% | 100.0% |
| S132 | 100.0% | 100.0% | 80.0% |
| S134 | 100.0% | 100.0% | 0.0% |
| S139 | 100.0% | 100.0% | 100.0% |
| S144 | 100.0% | 100.0% | 60.0% |
| S148 | 100.0% | 100.0% | 40.0% |
| Transferability[b] | 40/46 = 87.0% | 36/46 = 78.3% | 35/46 = 76.1% |

[a] Locality and voucher information are available in Appendix 1.

[b] Transferability = number of successfully cross-amplified loci/total number of microsatellites × 100%.

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APPENDIX 1.  Locality and voucher information for populations of *Smilax aspera*, *S. riparia*, *S. china*, and *S. hugeri* used in this study. Voucher specimens are deposited at the herbarium of Zhejiang University (HZU), Hangzhou, Zhejiang, China.

| Species | Population code | Voucher no. | Locality | Geographic coordinates | Altitude (m) | *n* |
|---|---|---|---|---|---|---|
| *Smilax aspera* L. | PL | HZU-0906014 | Lisbon, Portugal | 38°43′05″N, 09°11′24″W | 110 | 8 |
| | SM | HZU-906011 | Málaga, Spain | 36°38′52″N, 04°32′43″W | 250–300 | 8 |
| | IR | HZU-Q0906007 | Rome, Italy | 41°57′59″N, 12°48′18″E | 200 | 8 |
| | IS | HZU-Q0906003 | Sardinia, Italy | 39°12′59″N, 09°08′10″E | 100–150 | 8 |
| | GA | HZU-Q0906010 | Athens, Greece | 37°59′10″N, 23°49′24″E | 400–625 | 8 |
| | GC | HZU-Q0906011 | Chania, Greece | 35°30′59″N, 24°05′40″E | 150 | 8 |
| | TT | HZU-Z0906001 | Termessos, Turkey | 36°54′15″N, 30°30′11″E | 374 | 8 |
| | KL | HZU-Q10K001 | Lumuru, Kenya | 01°06′45″S, 36°40′57″E | 2189 | 8 |
| | SRL | HZU-F1012126 | Nuwara Eliya, Mahagasthota, Sri Lanka | 06°58′05″N, 80°45′38″E | 1900–2000 | 8 |
| | NS | HZU-BQ0908293 | Shivapuri, Nepal | 27°48′00″N, 85°22′00″E | 2000 | 8 |
| | CP | HZU-BQ0909326 | Pihe, China | 26°31′00″N, 98°55′00″E | 1050 | 8 |
| | CJ | HZU-BQ0908304 | Jilong, China | 28°19′00″N, 85°21′00″E | 1600–2000 | 8 |
| *Smilax riparia* A. DC. | | HZU-CY160344 | Hengyang, China | 27°16′33″N, 112°40′42″E | 1000 | 5 |
| *Smilax china* L. | | HZU-JXJ2016062604 | Wenzhou, China | 27°42′21″N, 119°40′30″E | 741 | 5 |
| *Smilax hugeri* (Small) J. B. Norton ex Pennell | | HZU-LP162465 | Chattahoochee, Florida, USA | 30°41′43″N, 85°08′46″W | 34 | 5 |

*Note*: *n* = number of individuals per population.