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.

Source: Applications in Plant Sciences, 5(4)

Published By: Botanical Society of America

URL: https://doi.org/10.3732/apps.1700005

Applications in Plant Sciences 2017 5(4): 1700005

PRIMER NOTE

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,

[1]Manuscript received 24 January 2017; revision accepted 7 March 2017.

The project was supported by the National Natural Science Foundation of China (grant no. 31400194, 31500184, 30830011), the Zhejiang Provincial Public Welfare Technology and Application Research Project (grant no. 2017C32044), the Science Foundation of Zhejiang Sci-Tech University (grant no. 14042010-Y), 521 Distinguished Young Scientist Foundation of Zhejiang Sci-Tech University (grant no. 11610132521509), and Basic Work Project of Ministry of Science and Technology (grant no. 2015FY110200).

[5]Authors for correspondence: liangzs@ms.iswc.ac.cn (Z.S.L.), panli@zju.edu.cn (P.L.)

doi:10.3732/apps.1700005

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	$(TTTTC)_5$	100	53	4	KY358008
	R: GCCACTACAACAACTTAGTGATTTTG					
S004	F: GCCCACTTTCATTGCCTTTA	$(TCA)_8$	111	53	15	KY358009
	R: AATGTGGGCGTGGTAAAAAG					
S006	F: AAAGGGGATGAGGAGAAGGA	$(AAG)_7$	133	59	9	KY358010
	R: AAACCACCATGACTCCTCCA					
S007	F: CTGCTTCCAGACAGAGGAGG	$(TGGTT)_5$	139	59	8	KY358011
	R: ACACTTCTTGGGTTGGCATC					
S009	F: GAGTGAGGAGGGAGGAGCTT	$(TC)_{33}$	159	58	23	KY358012
	R: CCGGAGAACCAGATGAAGAC					
S016	F: AGAACTTGAGGGTGTGTGGG	$(T)_{10}(TC)_6$	230	58	16	KY358013
	R: TTCATGCATACTTTTGCCGA					
S028	F: TAATCCCTCGCGAAATCAAG	$(GATC)_5$	120	53	3	KY358014
	R: CCCAAAATCGATCGAGAAAA					
S030	F: AAGCCAAGCAAACCCATTTA	$(GA)_{14}$	126	59	15	KY358015
	R: CACCCTCTGACTCCGAAGAG					
S034	F: CAGGGAGTTGGTCCTCAAAA	$(T)_{21}$	154	59	12	KY358016
	R: ATGGTTGCAAAGAAACACCC					
S046	F: CTAAGGCGATATCCTCAGCG	$(GTGGGC)_5$	226	59	7	KY358017
	R: CAGCCACTTGGTATCCACCT					
S049	F: AAGGGACATTTTTGTTCCCC	$(TAAA)_6$	248	59	4	KY358018
	R: GCAAGTTAAGCAACACAGTTAAGG					
S052	F: AGATCCACAGTTCCACCTGC	$(AAACTAT)_{10}$	266	59	8	KY358019
	R: GCGCTTGATGTGCTCAAATA					
S053	F: GATCTGGGTTTCTCGTTGGA	$(CTGGGA)_5$	269	59	6	KY358020
	R: GGCCATTTGGAAGAGACTGA					
S057	F: GAGATTTCCAGCAAAACCCA	$(CGAG)_4$	291	58	5	KY358021
	R: AGTTTCTGGGCCCTCTGTCT					
S060	F: CCATGGTGGACGACTTTCTT	$(GAT)_6$	311	59	3	KY358022
	R: GCATGGAAACGCCTATGATT					
S062	F: CTTGGCAACACCAATCAATG	$(TCCT)_7$	326	59	9	KY358023
	R: TGCACGTGATCACTGGATCT					
S063	F: CATTTCGATGAATCGTGTGG	$(CATCT)_5(TC)_{23}$	332	59	21	KY358024
	R: GTAGGGTTCGGTGCTGATGT					
S066	F: TCGATTTCCACCCATTTCTC	$(CGCCAC)_5$	354	59	10	KY358025
	R: GCTGAGTACTTGAGGGCGTC					
S072	F: CAGTGCCTCTTCCTTGCTTC	$(TGG)_5(GTGGCC)_3$	402	59	16	KY358026
	R: TATACCCAGGTCTCCGAACG					
S081	F: ATTTCGCCACTACCTTGCAC	$(CCCT)_6$	103	50	8	KY358027
	R: ATCCTTCATTCAATGCCGAG					
S083	F: GGACTGGATTCCGTTTTGCT	$(CCTCTA)_4$	105	50	4	KY358028
	R: AGCCAGGACATTGCCTTTAC					
S085	F: TGTTGGGTGAGCAAAACAAA	$(T)_{16}$	109	53	16	KY358029
	R: ACCTTTCTCCCCACTTGCTT					
S086	F: TAATTGGCTTCGGATTGACC	$(AG)_9$	112	50	28	KY358030
	R: GGAATTCGTTCTTCCCCATT					
S087	F: GGACTTGGTCATCAGGTCGT	$(TC)_{12}TAGGTC(TCGGA)_3$	116	55	21	KY358031
	R: TTGTGCAACCAAACTCCAGA					
S089	F: CACAAGCTTGATGAGGTCCA	$(TGGTT)_3$	125	53	7	KY358032
	R: AAGGACACGGACCATGAAAG					
S090	F: AGCAGCCTTGGGCTTATTTT	$(TAAAC)_3$	132	53	5	KY358033
	R: TTCTGTTGTGCGGATATTGG					
S093	F: GAAGGGAGGGAGGAGAAGTG	$(AG)_{12}$	135	53	7	KY358034
	R: CCGTTTAAAGATCCCGTCAA					
S094	F: TGCTGGAAGAACAACGACTG	$(GCTGTT)_4$	143	50	4	KY358035
	R: GTTACCGTTGGTCACCTGCT					
S096	F: TGGATTCATGTGTTTGGCTG	$(A)_{22}$	145	55	8	KY358036
	R: AAATCAGGCCTCCTCATTGTAA					
S097	F: CACCTTCTCCTCCTCTTCCC	$(TTC)_8$	148	51	9	KY358037
	R: TCATCTCCCCTCTTCTTCCC					
S100	F: CTGGAGATCTCACCCTCTCG	$(CCCTCT)_3$	155	50	6	KY358038
	R: CAATGAGACAGTCCGGATCA					
S104	F: AATTGGGATTTGATGATCGC	$(TC)_{17}$	168	53	11	KY358039
	R: CCAAAAACCCACGAGAGAAA					
S105	F: GCTGGTACTTCTTCTTGCCG	$(GGCGGA)_3$	168	55	6	KY358040
	R: ACTTCGAGAACAGCCTCCAA					
S110	F: TCACGTGTGAGGTTCTAGCG	$(AG)_7AA(AG)_{14}$	181	59	3	KY358041
	R: TGGCGTCCCAGTGAGTGT					
S113	F: ACGTAACTCTCGGTGCCATC	$(AG)_{11}$	185	55	5	KY358042
	R: CGTGTGGAAGGGAGGTAAAA					

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]

	Mediterranean group[b] ($N = 56$)				East African group[c] ($N = 8$)				South Asian group[d] ($N = 32$)			
Locus	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.

LITERATURE CITED

CHEN, C., Z. C. QI, X. H. XU, H. P. COMES, M. A. KOCH, X. J. JIN, C. X. FU, AND Y. X. QIU. 2014. Understanding the formation of Mediterranean-African-Asian disjunctions: Evidence for Miocene climate-driven vicariance and recent long-distance dispersal in the Tertiary relict *Smilax aspera* (Smilacaceae). *New Phytologist* 204: 243–255.

KALINOWSKI, S. T., M. L. TAPER, AND T. C. MARSHALL. 2007. Revising how the computer program CERVUS accommodates genotyping error increases success in paternity assignment. *Molecular Ecology* 16: 1099–1106.

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%.

KEARSE, M., R. MOIR, A. WILSON, S. STONESHAVAS, M. CHEUNG, S. STURROCK, S. BUXTON, ET AL. 2012. Geneious Basic: An integrated and extendable desktop software platform for the organization and analysis of sequence data. *Bioinformatics (Oxford, England)* 28: 1647–1649.

MAI, D. H. 1995. Tertiäre Vegetationsgeschichte Europas. Gustav Fischer Verlag, Jena, Germany.

MATASCI, N., L. H. HUNG, Z. YAN, E. J. CARPENTER, N. J. WICKETT, S. MIRARAB, N. NGUYEN, ET AL. 2014. Data access for the 1,000 Plants (1KP) project. *GigaScience* 3: doi:10.1186/2047-217X-3-17.

NARZARY, D., S. VERMA, K. S. MAHAR, AND T. S. RANA. 2015. A rapid and effective method for isolation of genomic DNA from small amount of silica-dried leaf tissues. *National Academy Science Letters* 38: 441–444.

ROUSSET, F. 2008. GENEPOP'007: A complete re-implementation of the GENEPOP software for Windows and Linux. *Molecular Ecology Resources* 8: 103–106.

ROZEN, S., AND H. SKALETSKY. 1999. Primer3 on the WWW for general users and for biologist programmers. *In* S. Misener and S. A. Krawetz [eds.], Methods in molecular biology, vol. 132: Bioinformatics: Methods and protocols, 365–386. Humana Press, Totowa, New Jersey, USA.

SAKAGUCHI, S., AND M. ITO. 2014. Development and characterization of EST-SSR markers for the *Solidago virgaurea* complex (Asteraceae) in the Japanese Archipelago. *Applications in Plant Sciences* 2: 1400035.

SCHUELKE, M. 2000. An economic method for the fluorescent labeling of PCR fragments. *Nature Biotechnology* 18: 233–234.

THIEL, T., W. MICHALEK, R. VARSHNEY, AND A. GRANER. 2003. Exploiting EST databases for the development and characterization of gene-derived SSR-markers in barley (*Hordeum vulgare* L.). *Theoretical and Applied Genetics* 106: 411–422.

XU, X. H., Y. WAN, Z. C. QI, Y. X. QIU, AND C. X. FU. 2011. Isolation of compound microsatellite markers for the common Mediterranean shrub *Smilax aspera* (Smilacaceae). *American Journal of Botany* 98: e64–e66.

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.