Development of Microsatellite Markers for Sargentodoxa cuneata (Lardizabalaceae) Using Next-Generation Sequencing Technology

Authors: Sun, Zhi-Xia, Ye, Lin-Jiang, Zhang, Fei, Hu, Wan, Fan, Deng-Mei, et al.

Source: Applications in Plant Sciences, 4(5)

Published By: Botanical Society of America

URL: https://doi.org/10.3732/apps.1600003

Applications in Plant Sciences 2016 4(5): 1600003

PRIMER NOTE

DEVELOPMENT OF MICROSATELLITE MARKERS FOR *SARGENTODOXA CUNEATA* (LARDIZABALACEAE) USING NEXT-GENERATION SEQUENCING TECHNOLOGY[1]

ZHI-XIA SUN[2,3], LIN-JIANG YE[3,4], FEI ZHANG[3], WAN HU[2,3], DENG-MEI FAN[3,5], AND ZHI-YONG ZHANG[3]

[2]College of Forestry, Jiangxi Agricultural University, Nanchang, Jiangxi 330045, People's Republic of China; [3]Laboratory of Subtropical Biodiversity, Jiangxi Agricultural University, Nanchang, Jiangxi 330045, People's Republic of China; and [4]Key Laboratory of Biodiversity and Biogeography, Kunming Institute of Botany, Chinese Academy of Sciences, Kunming, Yunnan 650201, People's Republic of China

- *Premise of the study:* Microsatellite loci were developed for a woody deciduous liana, *Sargentodoxa cuneata* (Lardizabalaceae), to help infer the evolutionary histories of ancient monotypic genera in subtropical China.
- *Methods and Results:* Using next-generation sequencing (Illumina MiSeq) technology, 21 polymorphic primer sets were identified in three wild populations. The number of alleles per locus ranged from one to seven. The expected and observed heterozygosities varied from 0 to 0.788 and 0 to 0.917, respectively. Transferability analyses were performed in *Stauntonia chinensis*, *Akebia trifoliata*, and *A. quinata*. Eighteen (85.7%), 18 (85.7%), and 17 (81.0%) markers were successfully amplified, respectively.
- *Conclusions:* The newly developed markers will facilitate further studies on genetic diversity and phylogeographic patterns throughout the distributional range of *S. cuneata*. This set of microsatellite primers represents the second report on molecular markers in Lardizabalaceae.

Key words: genetic diversity; Illumina MiSeq; Lardizabalaceae; microsatellite markers; *Sargentodoxa cuneata*.

Sargentodoxa Rehder & E. H. Wilson is a monotypic genus in Lardizabalaceae. The only extant member, *S. cuneata* (Oliv.) Rehder & E. H. Wilson, is a deciduous woody liana, with red stem sap and fleshy, dark blue berries. This species is mostly distributed in subtropical China and sporadically occurred in Laos and northern Vietnam (Chen and Tatemi, 2001). The species has wide ecological requirements and is not confined to a specific kind of vegetation (referred to as "generalists"). Therefore, *S. cuneata* may be an ideal species to infer the evolutionary histories of ancient monotypic genera in subtropical China, e.g., inferring refugial locations and the predominant pattern of migration that has led to their present geographical range (Tian et al., 2015).

In addition, *S. cuneata* is a valuable source of natural antioxidants because rich phenolic compounds can be extracted from its stems and roots (Krishnaiah et al., 2011). Traditionally, this species is used as a medicine (called Hongteng or Xueteng) in China to treat rheumatic arthritis, acute appendicitis, and painful menstruation (Tang et al., 2012). Furthermore, the long vines of *S. cuneata* are often cut to bundle objects. Due to long-term harvest, the natural resources of *S. cuneata* have been declining dramatically and it is now difficult to find individuals in flower and seedlings with diameters larger than 3–4 cm (Wang, 2007). To facilitate the conservation and sustainable use of wild genetic resources of *S. cuneata*, it is essential to understand patterns of genetic diversity, genetic structure, and gene flow among the natural populations. For this purpose, we applied next-generation sequencing technology (Illumina MiSeq sequencing) to develop a total of 21 microsatellite markers for *S. cuneata*, which may provide powerful tools for investigating the genetic diversity and spatial genetic structure of this species.

METHODS AND RESULTS

Leaf samples of 36 individuals were collected from three *S. cuneata* populations in subtropical China (12 from Xixiang, Shaanxi: accession no. JXAU35324; 12 from Tianmu Mountain, Zhejiang: accession no. JXAU35356; and 12 from Le'an, Jiangxi: accession no. JXAU35363) (Appendix 1). All samples were dried and preserved in silica gel. Genomic DNA was extracted from silica gel–dried leaves of *S. cuneata* using the cetyltrimethylammonium bromide (CTAB) method (Doyle and Doyle, 1987), and microsatellites were isolated using Illumina MiSeq sequencing technology (Illumina, San Diego, California, USA). A single individual from the Xixiang population was used to prepare the microsatellite-enriched library. Approximately 1 µg of genomic DNA was used as template for a library preparation with a NEBNext DNA Prep Master Mix Set for Illumina (New England Biolabs, Ipswich, Massachusetts, USA). The sequencing was performed on the MiSeq Benchtop Sequencer (Illumina) targeting 500-bp fragments using the 2 × 250-bp read length configuration. In this first run, 534,722 sequence reads with an average read length of 255 bp and a total of

[1]Manuscript received 8 January 2016; manuscript accepted 5 February 2016.

The authors thank S. Q. Lei and B. Li for help with the field survey and leaf collection, and Z. R. Zhang, Y. Yang, and J. Liu for help with laboratory work. This study was supported by grants from the National Natural Science Foundation of China (grant no. 31160043, 31160082) and the National Science and Technology Support Program (2012BAC11B02).

[5]Author for correspondence: dmf.625@163.com

doi:10.3732/apps.1600003

136,000 bases were obtained. These data were assembled using CLC Genomic Workbench (CLC bio, Aarhus, Denmark) into 80,760 contigs. QDD version 2.1 Beta (Meglecz et al., 2010) was then used to detect 1740 sequences with simple sequence repeats following the criteria: (1) only a single microsatellite locus present in each sequence; (2) the number of repeats should be greater than six; and (3) the flanking sequences of microsatellites should not be single-copy and must be long enough to design primers. One hundred forty-five sequences with long flanking regions were selected for primer design using Oligo 7.0 (Rychlik, 2007).

The newly designed PCR primer pairs were preliminarily screened for amplification using four samples (two individuals from each population). After optimization, the primer pairs were used to assess genetic polymorphism in 36 *S. cuneata* individuals. PCR amplifications were performed in a 20-μL reaction volume containing 50–100 ng of genomic DNA, 0.5 μM of each primer, 10 μL 2× *Taq* PCR MasterMix (0.1 unit *Taq* Polymerase/μL, 0.5 mM dNTP each, 20 mM Tris-HCl [pH 8.3], 100 mM KCl, and 3 mM $MgCl_2$; Tiangen Biotech, Beijing, China). PCR amplifications were conducted under the following conditions: an initial denaturing for 3 min at 95°C; followed by 32–35 cycles at 94°C for 45 s, at the annealing temperature for each specific primer (optimized for each locus, Table 1) for 45 s, and at 72°C for 45 s; and a final extension step at 72°C for 5 min. All PCR products were checked by electrophoresis on an 8% nondenaturing polyacrylamide gel and visualized with silver staining.

After amplification, 37 primers showed monomorphism (Appendix S1), and 21 primer pairs displayed polymorphisms and could be easily genotyped (Table 2). Standard genetic diversity parameters, e.g., the number of alleles per locus (A), expected heterozygosity (H_e), and observed heterozygosity (H_o), were calculated using GenAlEx version 6.5 (Peakall and Smouse, 2012). The probability of deviation from Hardy–Weinberg equilibrium (HWE) and linkage disequilibrium (LD) between all pairs of loci were tested using GENEPOP version 4.2 (Rousset, 2008). The number of alleles per locus ranged from one to seven, with a mean of 3.4. The expected and observed heterozygosities ranged from 0 to 0.788 and from 0 to 0.917, with averages of 0.546 and 0.276, respectively (Table 2). After the Bonferroni correction (Rice, 1989) had been performed, significant deviations from the HWE ($P < 0.05$) were seen for 10 loci in the Xixiang population, 11 loci in the Tianmu Mountain population, and seven loci in the Le'an population (Table 2). No significant linkage disequilibrium was detected between any pair of loci.

The marker transferability of the polymorphic primer pairs was tested upon three closely related species in Lardizabalaceae, *Stauntonia chinensis* DC., *Akebia trifoliata* (Thunb.) Koidz., and *A. quinata* (Houtt.) Decne. (five individuals for each species; Appendix 1), using the same PCR conditions as previously described. Eighteen markers (85.7%) were successfully amplified in *S. chinensis*, 18 (85.7%) in *A. trifoliata*, and 17 (81.0%) in *A. quinata* (Table 3).

TABLE 1. Characteristics of 21 microsatellite primers developed in *Sargentodoxa cuneata*.

Locus	Primer sequences (5′–3′)	Repeat motif	Allele size (bp)	T_a (°C)	GenBank accession no.
Sc_01	F: CAAAACCGAGAGACCCTGC	$(CT)_9$	153	52	KT727075
	R: AAATCACGAAAACGGGACC				
Sc_02	F: TGTGATATTCTGCATGG	$(CT)_8$	157	51.5	KT727076
	R: ATTACTCAGACAGGTTCCCA				
Sc_03	F: GGATTTGCGGTTGAACT	$(CT)_9$	238	48	KT727077
	R: TGGTGATAGTTCGCTGTA				
Sc_04	F: AGGTGGCTTGAAATAGTATC	$(TA)_8$	185	53	KT727078
	R: CCGCCACCAAAAAATCAA				
Sc_05	F: CTCCACAAGGCAAGTTT	$(TA)_{13}$	185	56	KT727079
	R: GGCTGTATCTTCCAACCT				
Sc_06	F: GAAGAGGCACCAAGGACA	$(CT)_8$	134	60.5	KT727080
	R: TTCTTTCAACATCCTCACAG				
Sc_07	F: CGCTGAATAAGATGGTTT	$(AT)_{19}$	274	49	KT727081
	R: AGATGAAAGAAACGATGC				
Sc_08	F: AACATACCCCAAGGAAAC	$(AC)_9$	254	53	KT727082
	R: TTGAAGGCGGGTAATACT				
Sc_09	F: AGGATGAGGGCAGGGTTTTT	$(CT)_9$	339	53	KT727083
	R: GGGAGGGGTATATTGTGGGT				
Sc_10	F: AGCCAAATAACAACAGTAAC	$(AT)_7$	213	53	KT727084
	R: TTCCCCATCACCATCTAT				
Sc_11	F: GTTTTGCGGGAGTTTATC	$(CT)_8$	186	54	KT727085
	R: CGCCCTTTTGGTACACA				
Sc_12	F: TATAACGCTCGGAACTTCT	$(AG)_9$	138	54	KT727086
	R: CAGGGACAATCATCATCA				
Sc_13	F: TGAAACTGGGAGCATTGA	$(AG)_7$	193	57	KT727087
	R: GCAACTTACCCTCAACATT				
Sc_14	F: TACCTGTTTCTGATACCCA	$(CT)_8$	152	57	KT727088
	R: CCTCAACCCACTTCAAATA				
Sc_15	F: ACATGAAAGATAAGCTCCCC	$(AG)_9$	92	56	KT727089
	R: TTCCTTAAATCACGCTTGCT				
Sc_16	F: GCCTGCTTAGACCAAAACCA	$(CT)_9$	125	51	KT727090
	R: TTTATGGGAGCTGAAGTTGA				
Sc_17	F: CTTCCAAATCTATGTGTGCT	$(CT)_8$	118	57	KT727091
	R: AGCCCTCTTCTCCCTAATCA				
Sc_18	F: GACTTGAGCGGGAAAACCAT	$(TC)_{10}$	113	57	KT727092
	R: TAGGAGACGAAACGAGTATC				
Sc_19	F: CCCTCACATATAAAACCGTC	$(CT)_8$	156	49	KT727093
	R: GTGAGATTGCCTGCATGTTT				
Sc_20	F: CGGAATTGGGGGGTTTTTTT	$(GA)_9$	205	47	KT727094
	R: CCAACCCAACCAACACCCTA				
Sc_21	F: GCTTGGTGAGGTTGGGAAT	$(AG)_9$	150	60	KT727095
	R: TATTAGCGGCGTGTCTTGA				

Note: T_a = annealing temperature.

TABLE 2. Results of initial primer screening in populations of *Sargentodoxa cuneata*.[a]

	Xixiang population (N = 12)				Tianmu Mountain population (N = 12)				Le'an population (N = 12)			
Locus	A	H_e	H_o	HWE[b]	A	H_e	H_o	HWE[b]	A	H_e	H_o	HWE[b]
Sc_01	3	0.288	0.333	1.000 ns	3	0.417	0.091	0.002*	2	0.278	0.000	0.006 ns
Sc_02	3	0.595	0.364	0.108 ns	4	0.653	0.500	0.104 ns	4	0.663	0.167	0.000*
Sc_03	2	0.444	0.000	0.001*	2	0.486	0.000	0.000*	2	0.298	0.182	0.281 ns
Sc_04	2	0.375	0.000	0.002*	4	0.642	0.417	0.021 ns	2	0.375	0.000	0.002*
Sc_05	3	0.517	0.182	0.009 ns	3	0.288	0.083	0.006 ns	2	0.500	0.000	0.000*
Sc_06	4	0.663	0.417	0.015 ns	4	0.622	0.417	0.005 ns	4	0.616	0.091	0.000*
Sc_07	4	0.580	0.333	0.007 ns	4	0.650	0.200	0.001*	3	0.632	0.091	0.000*
Sc_08	5	0.563	0.750	0.880 ns	4	0.510	0.667	0.794 ns	3	0.601	0.917	0.056 ns
Sc_09	4	0.653	0.167	0.000*	4	0.677	0.167	0.000*	3	0.559	0.333	0.025 ns
Sc_10	2	0.496	0.000	0.001*	3	0.517	0.364	0.292 ns	3	0.569	0.333	0.071 ns
Sc_11	6	0.756	0.545	0.005 ns	7	0.778	0.500	0.001*	5	0.760	0.083	0.000*
Sc_12	5	0.733	0.417	0.010*	6	0.726	0.250	0.000*	4	0.490	0.333	0.187 ns
Sc_13	4	0.707	0.273	0.000*	3	0.565	0.100	0.002*	3	0.635	0.083	0.000*
Sc_14	2	0.500	0.000	0.001*	3	0.645	0.000	0.000*	4	0.541	0.455	0.125 ns
Sc_15	3	0.497	0.083	0.001*	3	0.616	0.273	0.009 ns	3	0.663	0.417	0.078 ns
Sc_16	6	0.788	0.750	0.002*	5	0.781	0.417	0.000*	5	0.698	0.667	0.959 ns
Sc_17	1	0.000	0.000	NA	2	0.480	0.000	0.002*	1	0.000	0.000	NA
Sc_18	4	0.573	0.167	0.000*	3	0.645	0.545	0.003 ns	6	0.767	0.667	0.266 ns
Sc_19	3	0.635	0.250	0.012 ns	4	0.649	0.083	0.000*	3	0.541	0.364	0.222 ns
Sc_20	1	0.000	0.000	NA	3	0.344	0.250	0.400 ns	2	0.320	0.000	0.009 ns
Sc_21	4	0.677	0.750	0.094 ns	3	0.601	0.833	0.050 ns	4	0.545	0.273	0.014 ns

Note: A = number of alleles; H_e = expected heterozygosity; H_o = observed heterozygosity; HWE = Hardy–Weinberg equilibrium; N = number of individuals sampled.

[a] See Appendix 1 for voucher and locality information.

[b] Deviations from HWE were not statistically significant (ns) and statistically significant (*) after Bonferroni correction ($P < 0.05$); NA = not applicable (i.e., monomorphic locus).

CONCLUSIONS

The 21 polymorphic microsatellite loci will be useful in future molecular analyses of genetic diversity and genetic structure in *S. cuneata* populations and in elucidation of population demography. This is the second set of SSR markers developed for the Lardizabalaceae family, following the first report in *Akebia trifoliata* subsp. *australis* (Diels) T. Shimizu (Li et al., 2009). Amplification success for these markers in the transferability tests extends their potential usefulness to other genera in Lardizabalaceae.

TABLE 3. Cross-amplification results showing the number of alleles detected in 21 loci from *Sargentodoxa cuneata* in three related species.

Locus	*Stauntonia chinensis*	*Akebia trifoliata*	*Akebia quinata*
Sc_01	1	3	2
Sc_02	1	0	0
Sc_03	2	2	4
Sc_04	1	3	3
Sc_05	0	2	0
Sc_06	2	1	2
Sc_07	2	2	3
Sc_08	0	1	1
Sc_09	2	1	3
Sc_10	1	2	2
Sc_11	1	1	2
Sc_12	1	2	1
Sc_13	2	2	2
Sc_14	0	0	0
Sc_15	1	0	0
Sc_16	1	2	2
Sc_17	2	3	2
Sc_18	1	2	2
Sc_19	1	2	3
Sc_20	2	2	2
Sc_21	2	2	2

LITERATURE CITED

CHEN, D. Z., AND S. TATEMI. 2001. Lardizabalaceae. *In* Z. Y. Wu [ed.], Flora of China, vol. 6, 440–454. Science Press, Beijing, China, and Missouri Botanical Garden Press, St. Louis, Missouri, USA.

DOYLE, J. J., AND J. L. DOYLE. 1987. A rapid DNA isolation procedure for small quantities of fresh leaf tissue. *Phytochemical Bulletin* 19: 11–15.

KRISHNAIAH, D., R. SARBATLY, AND R. NITHYANANDAM. 2011. A review of the antioxidant potential of medicinal plant species. *Food and Bioproducts Processing* 89: 217–233.

LI, L., X. H. YAO, X. Z. CHEN, AND H. W. HANG. 2009. Development and characterization of microsatellite loci in Chinese medicinal plant *Akebia trifoliate* [sic] ssp. *australis* and cross-species amplification in closely related taxa. *Conservation Genetics* 10: 959–962.

MEGLECZ, E., C. COSTEDOAT, V. DUBUT, A. GILLES, T. MALAUSA, N. PECH, AND J. F. MARTIN. 2010. QDD: A user-friendly program to select microsatellite markers and design primers from large sequencing projects. *Bioinformatics* 26: 403–404.

PEAKALL, R., AND P. E. SMOUSE. 2012. GenAlEx 6.5: Genetic analysis in Excel. Population genetic software for teaching and research—an update. *Bioinformatics* 28: 2537–2539.

RICE, W. R. 1989. Analyzing tables of statistical tests. *Evolution* 43: 223–225.

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

RYCHLIK, W. 2007. OLIGO 7 primer analysis software. *Methods in Molecular Biology* 402: 35–59.

TANG, J., R. L. MA, Z. OUYANG, AND H. S. CHEN. 2012. Chemical constituents from the water-soluble fraction of wild *Sargentodoxa cuneata. Chinese Journal of Natural Medicines* 10: 115–118.

Tian, S., S. Q. Lei, W. Hu, L. L. Deng, B. Li, Q. L. Meng, D. E. Soltis, et al. 2015. Repeated range expansions and inter-/postglacial recolonization routes of *Sargentodoxa cuneata* (Oliv.) Rehd. et Wils. (Lardizabalaceae) in subtropical China revealed by chloroplast phylogeography. *Molecular Phylogenetics and Evolution* 85: 238–246.

Wang, H. F. 2007. *Sargentodoxa cuneata* in danger. *China Nature* 5: 54–55.

Appendix 1. Voucher and location information for populations of *Sargentodoxa cuneata* and species used for cross-amplification in this study. One voucher was collected for each population used; all vouchers were deposited in the herbarium of Jiangxi Agricultural University (JXAU), Nanchang, Jiangxi, China.

Species	Voucher specimen accession no.	Collection locality	Geographic coordinates	*N*
Sargentodoxa cuneata (Oliv.) Rehder & E. H. Wilson	JXAU35324	Xixiang, Shaanxi, China	32.8267°N, 107.8736°E	12
Sargentodoxa cuneata	JXAU35356	Tianmu Mountain, Zhejiang, China	30.3751°N, 119.4661°E	12
Sargentodoxa cuneata	JXAU35363	Le'an, Jiangxi, China	27.1994°N, 116.0033°E	12
Stauntonia chinensis DC.	JXAU36015	Garden of Jiangxi Agricultural University, Nanchang, China	28.7607°N, 115.8274°E	5
Akebia trifoliata (Thunb.) Koidz.	JXAU35822	Wugong Mountain, Jiangxi, China	34.2703°N, 108.2029°E	5
Akebia quinata (Houtt.) Decne.	JXAU35823	Wugong Mountain, Jiangxi, China	34.2703°N, 108.2029°E	5

Note: *N* = number of individuals sampled.