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异型花多态性微卫星标记的开发

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摘　要: 异型花是青藏高原特有的一年生草本植物。为了更好地利用和保护这一重要的植物资源, 本研究开发了异型花的多态性微卫星标记。首先, 基于限制性位点相关DNA测序(RAD-seq)技术, 开发了异型花14个多态性微卫星位点和2个单态性微卫星位点的引物; 随后, 对异型花的3个自然居群的57个个体的14个位点进行了分析。结果显示: 14个位点观察到的等位基因数为4~7个, 平均为5.071个; 期望杂合度范围为0.003~0.312 (平均值为0.194), 而观测杂合度范围为0~0.281 (平均值为0.047)。其中, 仅有3对引物在龙胆科的椭圆叶花锚中具有通用性。本研究开发的多态标记对研究异型花种群结构和遗传多样性具有重要意义。

关键词: 微卫星; 多态性; 群体遗传; 限制性位点相关DNA测序(RAD-seq); 异型花

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Development of Polymorphic Microsatellite Markers for *Sinoswertia tetraptera* (Gentianaceae)

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Abstract: *Sinoswertia tetraptera* (Maximowicz) T. N. Ho, S. W. Liu & J. Q. Liu is an annual herb endemic to the Qinghai-Tibet Plateau. Here, microsatellite (simple sequence repeat, SSR) markers for *S. tetraptera* were developed in order to better utilize and protect this essential medicinal plant resource. Primers for 14 polymorphic and 2 monomorphic microsatellite loci were obtained based on restriction site-associated DNA sequencing (RAD-seq). The 14 polymorphic loci were further investigated in 57 individuals from three natural populations of *S. tetraptera*. The number of alleles observed for these 14 loci ranged from 4 to 7 (mean: 5.071). The expected heterozygosity ranged from 0.003 to 0.312 (mean: 0.194), while the observed heterozygosity ranged from 0 to 0.281 (mean: 0.047). Three primers showed transferability to *Halenia elliptica*, a member of the subtribe Swertiinae (Gentianaceae). These polymorphic markers will be useful in future research on the population structure and genetic diversity of *S. tetraptera*.

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Key words: microsatellite; polymorphism; population genetics; restriction site-associated DNA sequencing (RAD-seq); *Sinoswertia tetraptera*

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Sinoswertia tetraptera (Maximowicz) T. N. Ho, S. W. Liu & J. Q. Liu, belonging to the subtribe Swertiinae (Gentianaceae), is an annual herb endemic to the Qinghai-Tibet Plateau (QTP)[1-3]. The seeds of *S. tetraptera* are mostly set through a cleistogamous pollination mode[1, 4]. This plant is a familiar traditional Tibetan herbal medicine also known as "Sang di", which is extensively used to treat liver and biliary diseases in China[5]. Taxonomically, *S. tetraptera* has been excluded from the genus *Swertia* and is closely related to the genus *Halenia* based on the molecular and embryological evidence[1, 6]. However, the systematic position of the species is tentative and needs further assessment[4].

Microsatellite (simple sequence repeat, SSR) markers have been widely applied in studying genetic diversity, population structure, and molecular identification due to their advantages including codominance, polymorphism, and reproducibility[7]. Though several microsatellite markers have been reported applicative in *S. tetraptera*, they are still insufficient for further research. Microsatellite markers have been developed in *Halenia elliptica* D. Don, and these microsatellite primers could be used in SSR amplification in the certain individuals of *S. tetraptera*[8]. However, only three pairs (X15, X23, and X42) of the primers proved effective in most of the individuals from three natural populations of *S. tetraptera*. The PCR products of primer X15 investigated in *S. tetraptera* were equally 500 bp, significantly larger than that expected in *H. elliptica* (216 bp), while primers X23 and X42 presented monomorphism in *S. tetraptera*. Thus, it is necessary to develop more polymorphic microsatellite markers for better utilization and conservation of *S. tetraptera* plant resources.

In a preliminary study, we scanned the general information on *S. tetraptera* microsatellites at a simplified genomic level based on the restriction site-associated DNA sequencing (RAD-seq). Four polymorphic microsatellite loci were found from 10 detected loci, which showed great feasibility and effectiveness[9]. In this study, the polymorphic microsatellite markers were developed accordingly for *S. tetraptera* using the RAD-seq database to investigate its genetic diversity in different populations, and the marker transferability of the polymorphic microsatellite primer pairs was tested in the closest relative of *S. tetraptera*.

1 Materials and methods

1.1 Sample collection and DNA extraction

The wild-growing specimens of *S. tetraptera* and *H. elliptica* from QTP were utilized as the sources of DNA in this study. The voucher specimens of the plants from different populations were deposited in the Herbarium of the Northwest Institute of Plateau Biology (HNWP), Chinese Academy of Sciences, Xining, China (Table 1). The genomic DNA was extracted from the silica gel-dried leaves using an improved cetyltrimethylammonium bromide (CTAB) method[10].

1.2 RAD-seq

Four genomic DNA samples were selected separately from four populations (DX, GL, LX and GZ) (Table 1), and then pooled. The subsequent library construction and high-throughput sequencing were completed by Novogene Corporation (China) according to the protocols described by Baird *et al*[11]. The pooled genomic DNA was quantified with Qubit (Invitrogen, Eugene, Oregon, USA) and then digested with *Eco*R Ⅰ. The *Eco*R Ⅰ-digested products were ligated to P1 adapter (Integrated DNA Technologies, Coralville, Iowa, USA), which contains an individual-specific index sequence of 6 bp for sample tracking. *Eco*R Ⅰ-based libraries were subsequently constructed from adapter-ligated fragment samples. The fragments were size-selected in a range from

Table 1 Information on the studied populations of *S. tetraptera* and *H. elliptica*

Species	Locality	Specimen	Coordinate	Altitude/m	Number
S. tetraptera	Dingxi (DX)	chen2013015	35°00′00"N, 103°59′17"E	2 550	21
S. tetraptera	Guoluo (GL)	chen2013161	32°51′19"N, 100°52′54"E	3 670	16
S. tetraptera	Linxia (LX)	chen2014008	35°34′31"N, 102°45′50"E	1 910	20
S. tetraptera	Ganzi (GZ)	chen2014190	31°52′06"N, 100°16′07"E	3 900	1
H. elliptica	Haibei (HB)	zhang2014351	37°46′50"N, 101°11′13"E	3 424	2
H. elliptica	Xining (XN)	zhang2014367	37°06′25"N, 101°42′22"E	2 650	2
H. elliptica	Linzhi (LZ)	chen2014320	29°14′00"N, 94°14′37"E	2 970	2
H. elliptica	Cuona (CN)	chen2014435	27°48′28"N, 91°59′30"E	3 720	2
H. elliptica	Linxia (LX)	zhang2014244	35°34′24"N, 102°46′51"E	3 139	2
H. elliptica	Huangnan (HN)	zhang2014174	34°26′45"N, 101°29′29"E	3 637	2
H. elliptica	Tongde (TD)	zhang2014116	34°48′55"N, 100°49′03"E	3 552	2
H. elliptica	Tongren (TR)	zhang2014145	35°16′31"N, 101°54′58"E	3 036	1
H. elliptica	Shigatse (SG)	chen2014521	27°25′44"N, 88°55′28"E	2 915	1

300 bp to 700 bp using a MinElute Gel Extraction kit (QIAGEN, Venlo, Netherlands). An adenine was added to the 3′ ends of the DNA fragments to create 3′-dA overhangs. The P2 adapter with a 3′-dT overhang was ligated to the ends of DNA fragments with 3′-dA overhangs in order to create an RAD-seq library template. Subsequently, a library enriched by high-fidelity PCR amplification was constructed. Finally, paired-end sequencing of the prepared RAD-tags with both adaptors was implemented on the Illumina MiSeq platform (Illumina, San Diego, California, USA; NCBI accession number: PRJNA392283).

1.3 Microsatellite loci identification and primer design

In the present study, microsatellites were considered to contain motifs of 2 to 6 nucleotides in size. The minimum length of microsatellite sequence was 12 bp. The upstream and downstream sequences of microsatellites were at least 100 bp in length, and the minimum distance between two microsatellites was 12 bp. The microsatellite loci were identified using the MISA tool (http://pgrc.ipk-gatersleben.de/misa/misa.html; 2018-09-20)[12]. Primers for amplification of these loci were designed using the Primer 3 (http://biotools.umassmed.edu/bioapps/primer3_www.cgi; 2018-09-24)[13]. All the designed primers matched the following conditions: 1) The length of primers ranges from 20 bp to 28 bp, and the optimum length is 24 bp; 2) The annealing temperature of each primer ranges from 60 ℃ to 65 ℃, and the optimum annealing temperature is 63 ℃; 3) The maximum annealing temperature difference between primer pairs is 1 ℃.

1.4 PCR and electrophoresis

An appropriate number of primers were randomly selected for amplification in the three natural populations (DX, LX and GL) (Table 1), and the amplified products were detected by electrophoresis. Polymerase chain reaction (PCR) amplification was performed in 20 μL reaction mixture containing 0.5 units of Taq polymerase (TaKaRa Biotechnology Co., Dalian, China), 2 μL 10× PCR buffer, 0.7 μL dNTPs (2.5 mmol/L each), 0.5 μL of each primer (10 μmol/L), and 1 μL of genomic DNA (15~25 ng/μL). The amplification of genomic DNA was carried out on Mastercycler Pro Thermal Cycler (Eppendorf Ltd., Germany). The PCR conditions comprised an initial denaturation step at 94 ℃ for 5 min, followed by 39 cycles of 94 ℃ for 40 s, appropriate annealing temperatures (Table 2) for 30 s, and 72 ℃ for 30 s, and then a final extension at 72 ℃ for 10 min. The PCR products were initially detected by 1.5% agarose gel electrophoresis, and then by 20% polyacrylamide gel electrophoresis (PAGE), with the DNA marker L (Sangon Biotech Co., Ltd., Shanghai, China). After electrophoresis, the PAGE gels were stained with ethidium bromide (EB) and pictures were taken using GelDoc 2000 with Quantity One software (Bio-Rad Laboratories, Inc., USA).

As *S. tetraptera* is systematically close to *H. elliptica*[1, 4, 14], polymorphic microsatellite primers for *S. tetraptera* were used to amplify the corresponding loci in *H. elliptica* (Table 1) under the same PCR

conditions as described above to test their universality.

2 Results and analysis

A total of 5 844 microsatellite loci with at least 100 bp at both ends were identified, of which 5 339 loci have had successfully designed PCR primers. Subsequently, 36 primers were randomly selected to represent the five microsatellite types (di-nucleotide, tri-nucleotide, tetra-nucleotide, penta-nucleotide, and hexa-nucleotide) (GenBank accession number: KY315138~KY315173). These primers were then screened using the total DNA isolated from the dried leaves of 57 *S. tetraptera* individuals from the three populations (DX, GL and LX) (Table 1). Amongst the selected microsatellite primers, 16 primer pairs were effective in amplification, although two pairs (for loci ST36 and ST37) were monomorphic (Table 2). The PAGE electropherograms of amplification with some primers are shown in Fig.1. The rest 20 primers failed in amplification.

For the 14 pairs of polymorphic primers, GENEPOP 4.4[15] was employed to calculate the total number of alleles per locus (A), deviations from Hardy-Weinberg equilibrium (HWE), expected heterozygosity (H_e), observed heterozygosity (H_o), null allele frequencies (N_A) and inbreeding coefficient (F_{is}).

GENEPOP analysis revealed that the number of alleles per polymorphic microsatellite locus ranged from 4 to 7, with a mean of 5.071 (Table 2). In all the populations, all the loci deviated from HWE ($P<0.01$). The expected and observed heterozygosity within the population ranged from 0.003 to 0.312 (mean: 0.194) and 0 to 0.281 (mean: 0.047), respectively (Table 3). Only ST11 and ST16 were detected to have highly significant linkage disequilibrium.

Cross-amplification of the 14 polymorphic microsatellite primers developed for *S. tetraptera* were performed in 16 individuals of *H. elliptica*. Three pairs of primers (for loci ST27, ST30 and ST41) were efficacious in amplification in *H. elliptica* (Table 4).

3 Discussion and conclusion

About two decades ago, the use of microsatellites in plants was limited by the cost of isolating large numbers of loci from target species[16]. Fortunately, in silico mining of microsatellites from sequence databases provides a series of efficient and inexpensive molecular approaches[17]. However, complete genome or transcriptome sequence data of *S. tetraptera* have not been obtained. Therefore, we used RAD-seq technology to perform reduced-representation sequencing, and fruitfully got a large number of microsatellite loci of *S. tetraptera*. Of the 36 randomly selected microsatellite primers, 16 were successful in amplification, and 14 of them showed

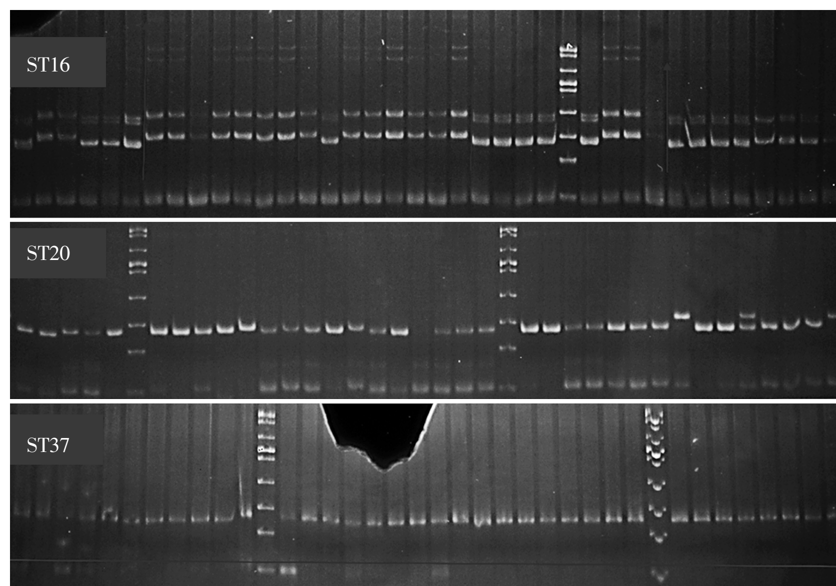

Fig.1 PAGE of PCR products with ST16, ST20 and ST37 primers

Table 2 Characterization of 14 polymorphic and 2 monomorphic microsatellite loci developed in *S. tetraptera*

Locus	Repeated motif	Primer sequence (5′–3′)	T_a/℃	A	Allele size/bp	GenBank accession number
ST11	(AGA)11	F: CAAGAAGTGGAGCTTCTGGTTTA R: AGCTTCCAATTCCTCATCTCTCT	53	5	131~149	KY315138
ST13	(AAATCA)6	F: TGATATCATAACCTATGGCCTGG R: AATTCAAATCCAAATCCAAAACC	55	4	103~121	KY315140
ST16	(TG)15	F: AAATGTTCTTTGATTCAACCAGG R: ACCTAGAAATCCTTGCTTTTTGG	55	7	143~155	KY315143
ST20	(TTTGT)6	F: GGTTATGGATACATGGGAAAACA R: ATAGGTGAGGATGGCATAAGGAT	55	5	138~158	KY315147
ST22	(AGA)10	F: CGAAAACTTACGGTATGGAAGTG R: ACTGCAATTATTTTTCTCTTCTCTCA	55	4	142~151	KY315149
ST23	(TC)14	F: GTCTGTTCTGATTTCATTCTCCG R: CATTGGAGAGCATATGGGTTAAG	53	7	109~121	KY315150
ST26	(ATA)11	F: CTTCAAAACTTCTCCCTCTCTCA R: ATAGGAAACAGGGAACTGGATGT	54	6	135~153	KY315153
ST27	(AATA)8	F: TTTTTAAATAGACGGTACGCAGC R: TTGCATTAGATGTAAGGTTAGTCACA	54	5	148~164	KY315154
ST30	(TTAT)7	F: GCTAGCCATTCGATTGAGGTAAT R: AGAACGCTAACCGTCATCATAGA	55	4	146~158	KY315157
ST31	(TTTTGT)6	F: AAAAGAAAACTTCAAAGGGCATC R: TCAAATTACATGGGTAAAAGGTCA	55	4	125~143	KY315158
ST35	(TGAA)7	F: TCATGTTAACGGTGTTGAGTTTG R: GATTACAACACCACTATGGGACC	55	6	145~165	KY315162
ST36*	(TCT)9	F: TTTTTGTCCTGCTCTGCTTTTT R: ATTCCTGTTGCGTTGTAATTGTT	55	1	124	KY315163
ST37*	(GA)13	F: ATCACCAACATTGGGTATCAGAG R: CATCGACAAGAAACAATCAATCA	55	1	150	KY315164
ST41	(AAAT)7	F: ACGATTGGTTCATTTAACACACC R: TGAACATTTTTGAAAGTCGGTTT	55	4	149~161	KY315168
ST42	(AG)12	F: CAAAGAACATAGCAATTTTGCAG R: ACGTATTGCTCTCTTTGTTGAGC	55	5	120~128	KY315169
ST46	(CA)8	F: TGAATGTCTAAATAAATGGGGGA R: GCACTCTATCACATATGAAATGCC	55	5	155~167	KY315173

Notes: T_a, annealing temperature; *, monomorphic microsatellite locus.

Table 3 Characteristics of 18 SSR primers developed for use in *S. tetraptera* at the population level

Locus	Dingxi (DX)					Guoluo (GL)					Linxia (LX)				
	A	H_o	H_e	F_{is}	N_A	A	H_o	H_e	F_{is}	N_A	A	H_o	H_e	F_{is}	N_A
ST11	4	0.228	0.256	0.113	0.167	4	0.158	0.180	0.125	0.080	3	0.281	0.238	–0.188	0
ST13	3	0.018	0.240	0.929	0.362	3	0.018	0.144	0.882	0.334	4	0	0.261	1.000	0.547
ST16	7	0	0.312	1.000	0.479	5	0	0.215	1.000	0.544	6	0	0.259	1.000	0.547
ST20	5	0.035	0.177	0.805	0.335	3	0	0.066	1.000	0.299	2	0	0.092	1.000	0.241
ST22	3	0.263	0.238	–0.107	0.176	2	0.123	0.114	–0.076	0.683	4	0.070	0.243	0.717	0.569
ST23	6	0.123	0.287	0.578	0.246	5	0.123	0.203	0.402	0.168	4	0.123	0.251	0.516	0.323
ST26	5	0.017	0.224	0.923	0.361	5	0.035	0.224	0.848	0.446	4	0	0.222	1.000	0.396
ST27	4	0	0.267	1.000	0.416	2	0	0.136	1.000	0.323	4	0	0.231	1.000	0.543
ST30	3	0	0.240	1.000	0.389	4	0	0.215	1.000	0.584	4	0	0.207	1.000	0.478
ST31	4	0.123	0.236	0.486	0.304	2	0	0.034	1.000	0.165	2	0	0.155	1.000	0.624
ST35	4	0	0.173	1.000	0.442	3	0	0.179	1.000	0.384	2	0	0.135	1.000	0.290
ST41	4	0	0.233	1.000	0.510	4	0	0.206	1.000	0.536	4	0	0.003	1.000	0.480
ST42	3	0.175	0.219	0.203	0.264	4	0	0.201	1.000	0.492	3	0	0.186	1.000	0.354
ST46	4	0	0.095	0.615	0.307	4	0	0.183	1.000	0.391	3	0.070	0.163	0.577	0.205

Notes: A, number of alleles per locus; H_e, expected heterozygosity; H_o, observed heterozygosity; F_{is}, inbreeding coefficient; N_A, null allele frequency.

polymorphism. It showed that it is feasible to develop microsatellite markers for *S. tetraptera* based on reduced-representation sequencing.

In all the populations, all the 14 polymorphic

Table 4 Cross-amplification of three polymorphic SSR markers developed for *S. tetraptera* in *H. elliptica*

Primer	HB	XN	LZ	CN	LX	HN	TD	TR	SG	Allele size/bp
ST27	1	1	1	1	1	1	1	1	1	152~160
ST30	1	1	1	1	1	1	1	1	1	150
ST41	1	1	1	1	1	1	1	1	0	153~157

Notes: 1, primers effective in amplification; 0, primers ineffective in amplification.

microsatellite loci deviated from HWE, which may be attributed to the main mode of cleistogamous pollination in *S. tetraptera*[4, 13]. The marker transferability of the polymorphic microsatellite primer pairs was tested in *H. elliptica*, showing that only 3 pairs of primers were universal in these two species. The results suggest that it is not advisable to use only one individual for universal detection of primers in *S. tetraptera* and its allies, which was also shown in previous studies[8].

Plant microsatellite markers have a wide range of applications[18]. However, a successful microsatellite-based study means heavy investment in terms of identifying loci, designing locus-specific primers and optimizing PCR conditions[19]. Therefore, this study has significant implications for reducing the cost of microsatellite-based research on *S. tetraptera*. In addition, 14 polymorphic and 2 monomorphic microsatellite primers for S. tetraptera have been developed based on the RAD-seq technique. These polymorphic markers and the microsatellite loci data set will be useful for *S. tetraptera* genetic study.

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