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NOTE

A rapid approach for SSR development in *Amorphophallus paeoniifolius* using RAD-seq

Yong GAO[1, #], Si YIN[2, #], Chao LIU[1], Haibo WANG[1], Honglong CHU[1], Lizhou TANG[1,*]

1. College of Biological Resource and Food Engineering, Center for Yunnan Plateau Biological Resources Protection and Utilization, Qujing Normal University, Qujing, Yunnan, 655011, China.
2. College of Biological Resource and Food Engineering, Qujing Normal University, Qujing, Yunnan, 655011, China.
[#] These authors contribute equally to this work. [*]Corresponding author's tel: +86-0874-8987890, e-mail: 124472623@qq.com.

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ABSTRACT: *Amorphophallus paeoniifolius* is a tuber plant distributed in Southeastern Asia. Methods of rapid and cost-effective molecular marker development are needed for population genetic studies of this species. Simple sequence repeats (SSRs) have been regarded as one of the most informative and versatile DNA markers. However, the traditional SSR development process is laborious and time-consuming. In this study, we reported an approach for SSR development in *A. paeoniifolius* based on paired-end restriction-site associated DNA sequencing (RAD-seq). A total of 1,537,924 high-quality RAD contigs were assembled, with the average sequence length of 340 bp, and 50,800 SSRs were identified. Ninety SSR primers were randomly selected and validated with 31 individuals of *A. paeoniifolius* and 16 individuals of *Amorphophallus kachinensis*. Twenty-three SSR loci were polymorphic in *A. paeoniifolius* and 13 loci in *A. kachinensis*. The overall number of alleles per locus ranged from two to seven. Compared with traditional approaches, our study yielded thousands of microsatellite loci in a short time with affordable costs. RAD-seq could be a new method for the development of SSR markers from non-model species.

KEY WORDS: *Amorphophallus paeoniifolius*, Polymorphism, RAD- seq, SSR development.

INTRODUCTION

Amorphophallus paeoniifolius (Dennst.) Nicolson is a tuber plant which belongs to the Araceae family. It is mainly distributed in Southeastern Asian countries, such as India, Thailand and China (Li *et al.*, 2010). It is considered as an important economic crop for the high production potential (Srinivas and Ramanathan, 2010). Vegetative propagation is usually used during the cultivation of *A. paeoniifolius*. The limited diversity of germplasms makes it vulnerable to bacterial diseases (Wu *et al.*, 2012). Developing molecular markers and gaining more information on the genetic diversity of wild resources of *A. paeoniifolius* could help to the genetic improvement of this plant. However, studies on population genetics of this plant are still in their infancy (Gao *et al.*, 2017).

Simple sequence repeats (SSRs) or microsatellites are considered as one of the most informative and versatile markers, and have been widely used in population genetics, genome mapping and quantitative trait loci identification in many species (Ekué et al., 2009). Despite of the advances in acquiring of single nucleotide polymorphism (SNP) data, SSR markers are still useful and more easily accessible for many studies, such as large-scale population genetic studies. The traditional SSR isolation approach, such as an enriched library followed by cloning and Sanger sequencing, is usually a costly and labor-intensive process. Methods of rapidly and cost-effectively microsatellite development are needed for population genetic studies of no-model species.

Next-generation sequencing (NGS) has provided an opportunity to characterize hundreds of SSRs quickly from organisms without reference genomes with affordable cost (Zalapa *et al.*, 2012). Zheng *et al.* (2013) developed sequence tag-derived simple sequence repeats (EST-SSRs) from transcriptome data of two *Amorphophallus* species (*A. konjac* and *A. bulbifer*). However, only a few molecular genetic markers are available for *A. paeoniifolius* so far (Santosa *et al.*, 2007).

Restriction site-associated DNA sequencing (RAD-seq) is a recently developed protocol for reduced representative genome sequencing (Davey *et al.*, 2011). Unlike other NGS assembling strategies, paired-end reads of RAD-seq were firstly clustered based on the reads with the restriction enzyme recognition site, and then the clustered reads were assembled (Hohenlohe et al., 2013). This strategy could improve the assembling accuracy, and therefore improve the success rate in the SSR development. For the pressing need of high resolution genetic data of this important plant, we report here on the development and validation of genomic microsatellite loci for *A. paeoniifolius* and its related species (*Amorphophallus kachinensis*) using RAD-seq.

Table 1 Locality information for ***Amorphophallus paeoniifolius*** **and** ***A. kachinensis.***

Population code	Species	Sample type	Location	*N*	Geographic coordinates	Altitude (m)
HHXJ[a,b]	*A. paeoniifolius*	Wild	Honghe, Yunnan, China	8	22°52'06.37"N, 103°34'03.07"E	146
MXXZ[a,b]	*A. paeoniifolius*	Wild	Xishuangbanna, Yunnan, China	8	21°52'41.53"N, 102°21'45.59"E	589
MBT[a,b]	*A. paeoniifolius*	Cultivated	Xishuangbanna, Yunnan, China	7	22°08'27.73"N, 100°55'46.96"E	1012
JD[a,b]	*A. paeoniifolius*	Cultivated	Simao, Yunnan, China	8	24°29'13.39"N, 100°49'36.64"E	1182
BNMN[a]	*A. paeoniifolius*	Cultivated	Xishuangbanna, Yunnan, China	4	22°09'11.34"N, 101°28'08.74"E	824
MLTS[a]	*A. paeoniifolius*	Cultivated	Xishuangbanna, Yunnan, China	1	21°26'00.61"N, 102°32'52.03"E	665
JDYSD[b]	*A. kachinensis*	Wild	Puer, Yunnan, China	8	24°40'41.53"N, 100°28'50.78"E	1050
MH[b]	*A. kachinensis*	Wild	Xishuangbanna, Yunnan, China	8	21°47'44.91"N, 102°22'55.58"E	973

Note: *N*: number of individuals sampled; [a] Populations used for RAD sequencing; [b] Populations used for validation of the SSRs.

MATERIALS AND METHODS

Plant materials

Two wild and two cultivated populations of *A. paeoniifolius*, and two wild populations of *A. kachinensis* were collected from southwestern China in 2016 (Table 1). DNA was extracted using a Plant Genomic DNA kit (Tiangen, Beijing, China).

DNA library preparation and sequencing

Genomic data of 36 *A. paeoniifolius* individuals were generated using RAD-seq. Sequencing library preparation and sequencing was conducted by Novogene Ltd. Briefly, the libraries were prepared following DNA digestion with *Eco*RI, and random fragmentation. Paired-end sequencing (PE 150) was conducted, and approximately 4 Gb of genomic data for each sample was generated by HiSeq 2000 (Illumina, USA). The raw RAD data was deposited in the National Center for Biotechnology Information (NCBI) Sequence-Read Archive (SRA) database under project ID PRJNA388361.

Sequence assembly and analysis

The raw data was firstly quality-filtered. Adapters and paired reads with low-quality bases or unidentified nucleotides were removed. The filtered data were then assembled using Stacks v1.44 (Catchen *et al.*, 2013). Briefly, the ustacks program was used to align reads with the restriction enzyme cutting site into putative loci; the minimum depth of coverage required (-m) was five and the maximum distance allowed between stacks (-M) was two. Next, the cstacks pipeline assembled these loci into catalogues, creating consensus loci, with a maximum distance between catalogue loci (-n) of two. Paired-end sequences were collated using the command 'sort_read_pairs.pl' in Stacks (Catchen *et al.*, 2013). The collated sets of reads were assembled by Velvet v1.1.05 (Zerbino and Birney, 2008) with a length cutoff of 200 bp.

Development of SSR markers for A. paeoniifolius

The MISA tool (Thiel *et al.*, 2003) was used to search for SSR motifs. The minimum number of repeats required to identify SSRs were: six for dinucleotides, and five for motifs that comprised three or more nucleotides. PCR Primers of each SSR locus were designed using Primer 3 (Rozen and Skaletsky, 2000).

Two wild and two cultivated populations of *A. paeoniifolius* (31 individuals) were selected to assess polymorphism of the microsatellite loci, and two wild populations of *A. kachinensis* (16 samples) were chosen to test the cross-compatibility. PCR reactions were performed using a volume of 15 μL , which included 7.5 μL of 2×PCR Mix (GeneStar, Beijing, China), 0.375 μL of 1 μM forward primer with a M13 tail (5′-CACGACGTTGTAAAACGAC-3′), 0.75 μL of 10 μM reverse primer, and 0.75 μL of 10 μM fluorescently labeled M13 primer (5' 6-FAM or HEX), 10－50 ng of template DNA. A touchdown PCR procedure was employed. The PCR conditions were as follows: an initial denaturation at 94°C for 5 min; 5 cycles at 94°C for 30 s, 62°C for 30 s, and 72°C for 30 s, with the annealing temperature decreasing 1°C per cycle; 30 cycles at 94°C for 30 s, 56°C for 30 s, and 72°C for 30 s; and a final extension at 72°C for 7 min. PCR products were analyzed on ABI 3730 (Life Technologies). Alleles were scored using GeneMarker 2.3 (Applied Biosystems). Basic genetic statistics, including the number of alleles (*A*), observed heterozygosity (H_O) and expected heterozygosity (H_E) were computed using GenAlex v6.5 (Peakall and Smouse, 2012). Departures from the Hardy–Weinberg equilibrium (HWE) were tested with FSTAT v2.9.3 (Goudet, 1995).

RESULTS

RAD sequencing and assembly of A. paeoniifolius contigs

A total of 166 Gb of RAD data were generated, and 161.0 Gb of clean data was retained after filtering. The assembled sequence comprised of 1,537,924 high-quality contigs (522Mb in total), with an average size of 339 bp and a contig N50 size of 340 bp (Table 2).

SSR development and characterization

A total of 50,800 SSRs were obtained using MISA software. Among them, the numbers of di-, tri-, tetra-, penta-, and hexa-nucleotide repeats were 36,414, 12,818, 1,222, 305, and 41, respectively. Repeat motifs of *A. paeoniifolius* SSR loci were analyzed. The most abundant repeat primitive is AG/CT, accounting for 33.0% of the total SSRs, followed by AT/AT (24.1%)

Table 2 Statistics of the assembled sequences of ***Amorphophallus paeoniifolius.***

Total sequences	Total bases (Mb)	Min length (bp)	Max length (bp)	Average length (bp)	Median length (bp)	N50 (bp)	(G+C)s
1,537,924	522.57	200	872	339.79	330	361	42.35%

Table 3 Characteristics of 25 microsatellite primer pairs developed in ***Amorphophallus paeoniifolius.***

Locus	Primer sequences (5' – 3')	Repeat motif	Product size range (bp)	Ta (°C)	GenBank accession no.
APssr1	F: GGCCCTCATCAACCCTAGAT R: TCAAGGGGCTCCAAGAATTT	(AG)12	144－152	62－56 (Touchdown)	MF805861
APssr2	F: TGAGCTGGATCAATATGAGGC R: ATGTGCTGCATAAGGCAGTG	(AG)13	317－339	62－56 (Touchdown)	MF805841
APssr3	F: GACTTGGGCCAGATCTTGAG R: TGTAGAAGCCTCACTGGAAGC	(AGCCA)6	148－178	62－56 (Touchdown)	MF805846
APssr4	F: TATGCATGCTCGCAAGAAAC R: CATTGCACCAAAGCACTTGT	(ATATC)6	268－303	62－56 (Touchdown)	MF805853
APssr5	F: ACTTTCGAGGAGTTGGATGG R: TTCTTGGGCACCCTATCAAG	(TAT)6	212－227	62－56 (Touchdown)	MF805865
APssr6	F: TCAGGCACTACAGCAAGTGG R: TTCTCCTCGTCTGTGCATTG	(GTCAA)5	195－205	62－56 (Touchdown)	MF805862
APssr7	F: TAGGGCTCATAGCCGAAGGT R: AATACCCATTCGTGGTGAGG	(CAG)8	268－303	62－56 (Touchdown)	MF805852
APssr8	F: TGCATCGACCTCACTGATTC R: CCATGGCCAGAGGATTAGAC	(TTC)7	167－184	62－56 (Touchdown)	MF805863
APssr9	F: AGACTCCTCGAGCCTTGGAT R: GCTTGCCCTTGACATGGTAT	(ATATT)5	197－252	62－56 (Touchdown)	MF805864
APssr10	F: TCCTGGAACATGTTGAATCCT R: TAGCAACAACTGGATGAGCG	(CT)12	222－232	62－56 (Touchdown)	MF805855
APssr11	F: TTTCGGGTTTTCACCTCAAC R: AGTCGGAGGGTAGGACCAAG	(CT)12	216－234	62－56 (Touchdown)	MF805858
APssr12	F: AAGGCCTTACACAGGCATTG R: AGGACACGCTCTTGGTCACT	(TAT)6	214－238	62－56 (Touchdown)	MF805856
APssr13	F: AACACGCACCAACACAAGAC R: GCTCACAGTAGCTGAACACTGTAT	(TG)12	334－346	62－56 (Touchdown)	MF805850
APssr14	F: GTCTCCATCAAGGGGAACAG R: TAGCGCATACGAAGCATACG	(CGC)7	227－239	62－56 (Touchdown)	MF805844
APssr15	F: CGACCACCGGGTTACTATTG R: CACGAACCTCGAGGAAGAAC	(GTT)6	169－187	62－56 (Touchdown)	MF805843
APssr16	F: GCCATGTAGATACTCCTCTCTCC R: AGATTTCTGTCTCCGCGTGT	(CTT)9	250－268	62－56 (Touchdown)	MF805847
APssr17	F: CACATGCACACACCATTTTG R: GCACCTTGGTTGTTAAGCCT	(TG)11	168－178	62－56 (Touchdown)	MF805842
APssr18	F: TCTGTGTGCTGAGCTATGGG R: GATGAGCTTGCTTCCCTTTG	(TTA)7	232－241	62－56 (Touchdown)	MF805854
APssr19	F: TCACAGTCCGAATCGAACAC R: GAGGGTCATGTTCCATGCTT	(GCA)7	201－210	62－56 (Touchdown)	MF805860
APssr20	F: GGTGGGACTCACGTGTCAAT R: CGCCGCCAAATTTTATATGT	(CT)10	223－227	62－56 (Touchdown)	MF805845
APssr21	F: GAATGGAATGCAAGATGCAC R: CATGCTCACAAGTTGCATTG	(GTTT)7	253－273	62－56 (Touchdown)	MF805857
APssr22	F: AGCAGCCCATCATATTTTCG R: TGCGCCATGTTACTTACTGC	(CT)14	214－236	62－56 (Touchdown)	MF805848
APssr23	F: CACACAGGTTGTGATGAACG R: TACCATTTGGAGACCCGAAG	(TC)8	209－221	62－56 (Touchdown)	MF805851
APssr24	F: GCATGGAAGATGCATGTGTG R: GGATCGTTCATCGTCCAGTT	(TTAA)5	165－169	62－56 (Touchdown)	MF805859
APssr25	F: GAGGCCATGAGTGTTCTGGT R: TGAAATGGCTGTGATTGCAT	(AT)13	298－314	62－56 (Touchdown)	MF805849

NOTE: Ta: annealing temperature

(Fig. 1). For all SSRs, 12,430 primer pairs were successfully designed using Primer 3.

Ninety primer pairs were randomly selected to evaluate the amplification rate and polymorphism of *A. paeoniifolius* samples. Thirty-one primer pairs failed to amplify. For the remaining primers, 23 loci were polymorphic in *A. paeoniifolius*, and 15 loci could be amplified and 13 loci were polymorphic in *A. kachinensis*. The overall number of alleles per locus ranged from two (APssr6, APssr18, APssr19, APssr20 and APssr24) to seven (APssr4, APssr13 and APssr25), with an average of four alleles per locus. The H_E values for each locus within the population ranged from 0.000 to 0.727, and the H_O values varied from 0.000 to 1.000 (Table 3 and Table 4). No sign of departure from HWE was detected for all loci after Bonferroni correction.

Table 4 Genetic properties of the newly developed 25 microsatellites of ***Amorphophallus paeoniifolius*** and ***A. kachinensis.***

	HHXJ (n=8)			MXXZ (n=8)			MBT (n=7)			JD (n=8)			JDYSD (n=8)			XM (n=8)		
Locus	*A*	H_O	H_E	*A*	H_O	H_E	*A*	H_O	H_E	*A*	H_O	H_E	*A*	H_O	H_E	*A*	H_O	H_E
APssr1	2	0.500	0.375	2	0.375	0.305	2	1.000	0.500^M	2	0.125	0.117	3	1.000	0.555	1	0.000	0.000^M
APssr2	3	0.875	0.539	2	1.000	0.500^M	2	1.000	0.500^M	2	0.500	0.375	3	0.167	0.486	1	0.000	0.000^M
APssr3	2	0.125	0.117	2	0.125	0.117	2	0.000	0.245	2	0.375	0.305	2	0.143	0.337	2	0.167	0.153
APssr4	2	1.000	0.500^M	2	1.000	0.500^M	4	1.000	0.622	2	1.000	0.500^M	3	0.750	0.594	3	0.857	0.571
APssr5	1	0.000	0.000^M	2	0.250	0.219	3	0.571	0.439	2	0.571	0.408	2	1.000	0.500^M	2	1.000	0.500^M
APssr6	2	0.875	0.492	2	0.500	0.375	2	0.286	0.245	2	0.875	0.492	2	1.000	0.500^M	2	1.000	0.500^M
APssr7	2	0.750	0.469	2	1.000	0.500^M	2	1.000	0.500^M	2	0.125	0.117	3	1.000	0.625	1	0.000	0.000^M
APssr8	2	0.625	0.430	2	0.125	0.117	2	0.714	0.459	2	0.125	0.117	1	0.000	0.000^M	2	0.250	0.219
APssr9	2	0.500	0.375	3	0.625	0.461	2	0.857	0.490	2	0.125	0.117	2	1.000	0.500^M	2	0.200	0.180
APssr10	2	0.375	0.305	2	0.500	0.375	1	0.000	0.000^M	2	0.875	0.492	3	0.167	0.569	2	0.500	0.375
APssr11	3	1.000	0.555	2	0.750	0.469	2	0.857	0.490	2	1.000	0.500^M	4	1.000	0.742	4	0.857	0.582
APssr12	2	0.500	0.375	1	0.000	0.000^M	2	0.714	0.459	1	0.000	0.000^M	3	0.875	0.570	2	0.125	0.117
APssr13	5	0.875	0.727	4	0.750	0.711	2	1.000	0.500^M	2	1.000	0.500^M	3	0.375	0.320	2	0.125	0.305
APssr14	1	0.000	0.000^M	1	0.000	0.000^M	1	0.000	0.000^M	1	0.000	0.000^M	1	0.000	0.000^M	2	0.000	0.278
APssr15	2	1.000	0.500^M	2	1.000	0.500^M	2	1.000	0.500^M	2	1.000	0.500^M	2	0.875	0.492	3	1.000	0.625
APssr16	3	0.714	0.602	2	0.143	0.133	2	1.000	0.500^M	3	0.571	0.439	—	—	—	—	—	—
APssr17	3	0.167	0.542	3	0.000	0.560	2	1.000	0.500^M	2	0.500	0.375	—	—	—	—	—	—
APssr18	2	0.875	0.492	2	0.250	0.219	2	1.000	0.500^M	2	1.000	0.500^M	—	—	—	—	—	—
APssr19	2	0.625	0.430	2	0.875	0.492	2	0.429	0.337	2	0.875	0.492	—	—	—	—	—	—
APssr20	2	0.375	0.430	1	0.000	0.000^M	2	1.000	0.500^M	2	1.000	0.500^M	—	—	—	—	—	—
APssr21	5	0.667	0.667	1	0.000	0.000^M	2	0.857	0.490	2	1.000	0.500^M	—	—	—	—	—	—
APssr22	3	0.750	0.617	3	0.857	0.643	1	0.000	0.000^M	1	0.000	0.000^M	—	—	—	—	—	—
APssr23	4	1.000	0.727	4	0.875	0.672	3	1.000	0.561	3	0.750	0.539	—	—	—	—	—	—
APssr24	2	0.500	0.469	1	0.000	0.000^M	1	0.000	0.000^M	1	0.000	0.000^M	—	—	—	—	—	—
APssr25	5	0.714	0.704	2	0.667	0.444	2	0.667	0.444	3	0.500	0.403	—	—	—	—	—	—

NOTE: n: number of individuals; *A*: number of alleles; H_O: observed heterozygosity; H_E: expected heterozygosity; —: Primers failed to amplify; M: monomorphic.

DISCUSSION

In this study, we assembled a large number of contigs of *A. paeoniifolius* within a relatively short period. And 50,800 SSRs have been discovered from these sequences. With sequencing costs decreasing in the NGS, the procedure of RAD-seq (RAD library construction and Illumina sequencing) can be completed in 2-4 weeks with affordable costs ($ 110 for 4Gb data), while the traditional SSR isolation protocols usually takes about more than one month (library construction, cloning and Sanger sequencing) (Xue *et al.*, 2017). Moreover, our approach is able to identify thousands of SSRs at a time. All these results suggested that RAD-seq was an effective way to develop genomic SSR markers from non-model organisms such as *A. paeoniifolius*.

Out of 90 primer pairs assessed, 59 pairs (65.6%) yielded amplicons with expected sizes. It was identical to the amplification rate (60-90%) of previous studies (Liang *et al.*, 2009; Wang *et al.*, 2011), which implied that the SSR makers developed by RAD-seq were valid. We obtained 24 polymorphic markers in *A. paeoniifolius*, and 13 polymorphic loci in *A. kachinensis* (Table 4). The polymorphic proportion was 26.7% (*A. paeoniifolius*) and 14.4% (*A. kachinensis*), respectively. This ratio was comparable with other plants (Peakall *et al.*, 1998; Varshney *et al.*, 2005).

In conclusion, an advance in the identification of large numbers of SSR loci in *A. paeoniifolius* based on RAD-seq was reported, and a total of 12,430 primer pairs were successfully designed (Table S1). These SSR markers also showed good cross-species transferability in *A. kachinensis* and could be used in genetic studies of other related *Amorphophallus* species. The SSR loci identified in our study could be further developed for genetic studies of the *A. paeoniifolius* germplasm.

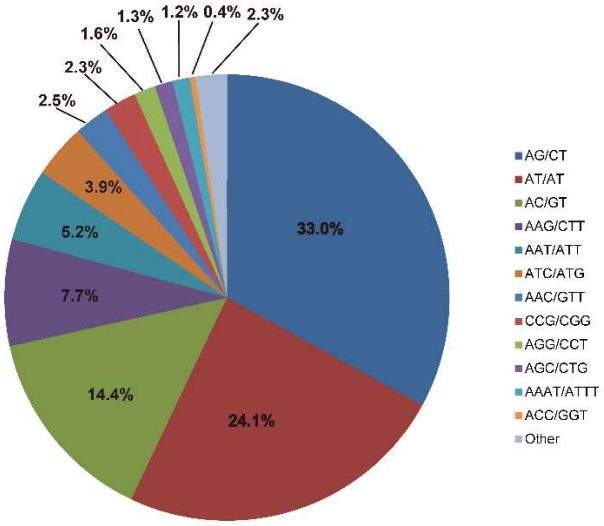

Fig. 1 Percentage of different SSR repeat motifs of ***A. paeoniifolius.***

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