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Full Length Research Paper

Isolation and characterization of EST-SSRs in the Ramie

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In order to develop the more EST-SSRs, 320 ESTs of ramie from NCBI were analyzed. 76 SSR primer-pairs developed from 320 ESTs were tested among 62 ramie individuals, and 27 SSR loci were polymorphic. Among the 27 primers, the numbers of alleles per locus ranged from two to five, in which 19 primers amplify two alleles, seven primers amplify three alleles, and only one primer amplifies five alleles. Observed heterozygosity (H_O) of overall loci among the 27 primer pairs ranged from 0.16 to 0.93 and expected heterozygosity(H_E) ranged from 0.21 to 0.66.Of the 27 loci, 14 displayed significant deviations from Hardy–Weinberg expectations (P < 0.05). No significant linkage disequilibrium (LD) was detected among the 27 loci. The 27 primers have been used in the ramie molecular linkage map and genetic diversity analysis successfully.

Key words: Microsatellite marker, ramie, EST, EST-SSR.

INTRODUCTION

The ramie, also called Chinese Grass, one of the oldest fibre crops, has been used in fabric production for six thousand years (Xiong, 2008). At the same time, it has not only been used in the soil and water conservation in the Changjiang River region, but also as a forage crop in China (Xiong, 2010). As an important crop, it is necessary to do QTL mapping or genetic diversity analysis by using of molecular markers.

SSRs or microsatellites are tandemly repeated short DNA units (1 to 6 bp long) which may range in the number of repeats at a special locus. SSRs are easy to use and analyze (Morgante and Olivieri 1993). Traditional experimental methods for developing SSRs are based on isolating and sequencing clones containing putative SSR tracts, together with designing and testing flanking primers, so it is expensive using this way. Recent studies have revealed that gene transcripts also contain repeat motifs, and the abundance of expressed sequence tags (ESTs) is an attractive potential library of microsatellite markers (Kantety et al., 2002). In order to reduce costs, the EST-SSR method was developed and EST-SSRs have been identified in many species, such as wheat (Eujayl, 2002), cotton (Han et al., 2006), Gauffre (2006), Zhou et al., (2005). In the ramie, Xing (2008) has also developed 8 EST-SSRs.

In order to develop the more EST-SSRs, 320 ESTs of the ramie from NCBI were analyzed. Here we report the isolation and characterization of microsatellite loci, most of them are first published for this species.

MATERIALS AND METHODS

EST-SSR identification and primer design

320 ESTs of ramie from NCBI were analyzed by using the simple sequence repeat identification tool (SSRIT) (http://www.gramene.org/db/searches/ssrtool). The sequences which contained clear repeated units (mono, di, tri, tetra, penta, or hexanucleotide) were selected to evaluate the polymorphism and mononucleotide, dinucleotide, trinucleotide, tetranucleotide, pentanucleotide and hexanucleotide repeats for at least 16, 6, 4, 3, 3 and 2 times, respectively. The EST- SSR markers ["ibfc" (Institute of Bast Fiber Crops, Chinese Academic of Agricultural Science)], as a short prefix, were designed with the primer 5 software.

The major parameters for primer design were set as below:

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Table 1. Characterization of 27 microsatellite loci derived from ramie EST , described by locus name, forward (F) and reverse (R)primer sequences, repeat motif, optimal annealing temperatures (T_a), size of allele (Size), number of alleles (N_A), observed heterozygosity (H_O), expected heterozygosity (H_E) and significance of departure from Hardy–Weinberg equilibrium (HWE). Polymorphism statistics were performed on 62 individuals.

Locus	Repeat	Primer sequences (5'-3')	GenBank Accession no.	Size (bp)	Ta (°C)	N_A	H_O	H_E	HWE *P.* value
ibfc51	(CCG)4	F: AAGACGAAGTGCGACGAC R: CTCCTCTTCTCCACCTCCTC	ES584548	122	60	2	0.53	0.49	0.60
ibfc50	(AAT)9	F: AACAATCCAGGAGTGGCAATC R: ACAAGCGAAGATCGTCTCATC	ES584549.1	157	50	2	0.39	0.48	0.14
ibfc35	(CTC)4	F: CGTTCAGTCACCAGCAAGG R: GAGGGAAGCAGGGAGAGC	FG588844.1	165	60	3	0.73	0.53	0.00
ibfc38	(CTT)4	F: TAATCCCTCAATGGCTCTTTTC R: GAGAAGGATACGAATTGACAGG	FG588827.1	196	51	2	0.66	0.46	0.00
ibfc40	(TTA)4	F:TGTATAGAACTGAGTAAATGATTG R: CAACTTTCTTAAACCACTTTCG	FG588929.1	147		3	0.42	0.46	0.09
ibfc43	(CTT)4	F: CGAGCCTTCTTCTTCTTCTGG R: GCAAGCAATACGGACAGTAGG	FG588901.1	152	50	2	0.37	0.30	0.02
ibfc10	(T)18	F: CGTGAAAATAGTGATATGTGTG R: ACTGTAACAATCAAGAAGAAACC	ES584587.1	113	52	2	0.53	0.50	0.64
ibfc19	(AG)7	F: GCCACAGCCGAGGAAGAG R: TCTCATCACCACCACCTTAGG	FG588886.1	95	55	3	0.93	0.50	0.00
ibfc27	(GT)6	F: AGCCAGGTTCCAGAAGTCC R: CATAATCACAAAGTCTCGGTTCC	ES584570.1	132	56	2	0.45	0.49	0.58
ibfc76	(CGAGCA)3	F: TCCACAACGGCGAAGATG R: GTGATGTTCCAATAGTCCAACC	EH667197.1	172	55	2	0.26	0.25	0.79
ibfc28	(TGA)5	F: TCCCACCACGGACTACTG R: AACCACCATCATCATCATCATC	GO653159.1	140	55	5	0.29	0.41	0.02
ibfc11	(T)16	F: GCGGAGGCTTAATTTGCTTTG R: ACTCAATACATACACGGCACTAG	ES584571.1	122	51	2	0.32	0.50	0.00

Table 1. Contd.

ibfc16	(TG)15	F: ACCTCTACGGACCTCTTCTTC R:CATAACATAACATGACACACAAGC	FG588840.1	155	52	2	0.56	0.48	0.21
ibfc24	(GT)6	F: GAGCCAGAGCCAGGTTCC R: ACAAAGTCTCGGTTCCTTACAC	ES584580.1	132	55	2	0.51	0.50	0.84
ibfc34	(TAT)4	F: AATAGAATGTGGAGGCGATAGAG R: AAACCATAAATCAACTACCGAACC	FG588845.1	183	53	2	0.27	0.43	0.00
ibfc64	(CTTT)3	F: CTTGAGATACAGCCTTCCATTAG R: CACACCTCGCTTCCCTTG	FG588824.1	130	55	2	0.24	0.21	0.16
ibfc63	(CTTC)6	F: TTCTTCCTTCCTTCCTTCCTTC R: TTGTGGCTGGTGATACTGAG	FG588834.1	95	56	2	0.82	0.49	0.00
ibfc65	(AAGA)3	F: ACGAACCACAACACAGAGAG R: ACGAGGGAACACCAGAGAG	FG588888.1	90	60	3	0.41	0.48	0.00
ibfc62	(TTGG)3	F: GAAACTATTTCCACCAACAAAG R: ACACACATTCCTACACACC	GH571858.1	186	54	2	0.39	0.31	0.07
ibfc69	(GAAGT)3	F: AAGCCGAGCGTGAAGAAG R: ACACACAGAAAGAACACAAGAC	FG588842.1	103	54	2	0.84	0.62	0.00
ibfc53	(TTC)7	F: GGCTCAAGTTTGCTCATAGATTC R: CGGCTTCGCTTTAGGATTTG	EH667249.1	124	56	3	0.62	0.59	0.14
ibfc56	(TCG)5	F: CGGTCTGTGGATACGAATGG R: GACGACGACGACGATGATG	EH667229.1	124	61	3	0.49	0.61	0.28
ibfc20	(TA)12	F: AGTGCGGAGATAACTGTTC R: GGCTACTTTATTCTAAACCAAAC	FG588867.1	191	54	3	0.44	0.66	0.01
ibfc57	(GTC)6	F: CGGATATGGTGGAGGTTATGC R: CAGAACGACGACGACGAC	EH667220.1	147	54	2	0.68	0.49	0.01

Table 1. Contd.

ibfc75	(ATTTTT)3	F: GACTAGACATTTCAAATAGCCTTC R: AAGAATACACCTGATGGAGATAC	GH571857.1	109	53	2	0.16	0.40	0.00
ibfc74	(GAAGCC)3	F: CTGAAGCCGAAGCCGAAG R: TTCCACTGCTGCCTCCTC	FG588845.1	101	58	2	0.25	0.48	0.00
ibfc72	(ACCGAA)3	F: GCAGCCGTTGAGGAAGAG R: CCTTGTCCGCCGATTTGG	GO653138.1	82	58	2	0.62	0.50	0.12

primer length 18 to 24 bp with 20 bp as the optimum; PCR product size 80 to 200 bp; optimum annealing temperature 52°C; GC content 35 - 60% with 50% as the optimum. The primers were synthesized by Bioasia Biotech, Shanghai, China.

PCR amplification, electrophoresis and primer evaluation

Young leaves of ramie were used for DNA extraction following the protocol of Tiangen DNA extraction kit. PCR reactions were carried out in 10µl reaction volumes with 1×PCR buffer, 0.2 mM dNTP, 1U Taq DNA polymerase (Tiangen), 0.5 µM each primer and 0.5 µl DNA under the following PCR conditions, which were 5 min at 94°C, followed by 30 cycles of 30 s at 95°C, 30 s at the primer-specific annealing temperature, 30 s at 72°C, and a final extension of 10 min at 72°C. The PCR products were separated on 8% polyacrylamide gels using electrophoresis, and silver dyeing was conducted according to Zhang et al. (2000).

Characterization of EST-SSR

Observed heterozygosity (H_O), expected heterozygosity (H_E), significant deviations from hardy–weinberg equilibrium (HWE) and tests for linkage disequilibrium were performed by using Popgen32.

RESULTS AND DISCUSSION

The clear repeated units were found in 76 EST-SSRs, which meets our standard. 76 primer-pairs were tested among 62 ramie DNA and PCR products of 50 pairs were effectively amplified. Among the 50 pairs, 27 pairs obtain clear and polymorphic PCR bands. Table 1 lists the summary data for the 27 characterized polymorphism. Among the 27 primers, the numbers of alleles per locus ranged from two to five, in which 19 primers amplify two alleles, seven primers amplify three alleles, and only one primer amplified five alleles. Observed heterozygosity (H_O) of overall loci among the 27 primer pairs ranged from 0.16 to 0.93 and expected heterozygosity (H_E) ranged from 0.21 to 0.66. Of the 27 loci, 14 displayed significant deviations from Hardy–Weinberg expectations ($P < 0.05$). No significant linkage disequilibrium (LD) was detected among the 27 loci.

Xing (2008) designed 8 EST-SSRs in ramie, and we compared his results with our SSR primers, which showed that ibfc 50 and ibfc 53 maybe the same to the two SSR primers developed by Xing due to the same repeat motif, but it could not been confirmed since Xing only provided the SSR primer sequence, and does not list the accession number of GenBank of the EST.

In conclusion, 27 SSR primers from EST of ramie were developed in this study. These primers have been used in other geographic populations of ramie successfully in our lab and now those primers are using in the ramie molecular linkage map and genetic diversity analysis, which will give a better understanding for the ramie.

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