Development of simple sequence repeats (SSR) markers of ramie and comparison of SSR and inter-SSR marker systems*

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Abstract Ramie (*Boehmeria nivea* L.) is an important bast fiber crop. To study genetic background of this species, we isolated and characterized microsatellite markers of ramie. A genomic library containing inserts of rapid amplification of polymorphic DNA (RAPD) fragments was constructed, and screened by PCR amplification using anchored simple sequence repeats as primers. A total of 26 clones were identified as positives, and 13 microsatellite loci were found after sequencing. The polymorphism of these 13 microsatellite loci was examined and the utility of simple sequence repeats (SSR) and inter-SSR (ISSR) marker systems for genetic characterization compared using 19 selected ramie cultivars. Both approaches successfully discriminated the 19 cultivars which differed in the amount of polymorphism detected. The level of polymorphism detected by SSR was 95.0%, higher than that by ISSR (72.3%), but the average polymorphism information content (PIC) of ISSR (0.651) was higher than that of SSR (0.441). The higher PIC value of ISSR suggests that ISSR is more efficient for fingerprinting ramie cultivars than SSR markers. However, because the SSR loci are codominant, they are more suitable for determining the homozygosity levels of ramie, constructing linkage map, quantitative trait loci study of complex traits and marker-assisted selection.

Keywords: ramie (*Boehmeria nivea* L. Gaud), simple sequence repeats, inter-simple sequence repeats.

Ramie (*Boehmeria nivea* L. Gaud), a perennial herb, has been grown as fiber crop for many centuries, and its main producing countries are China, Brazil, Philippines, India, South Korea and Thailand[1]. The bast fiber from its woody stem is very durable, pure white in color and has a silky luster. Ramie fiber is widely used in fine linen and other clothing fabrics, upholstery, canvas, filter cloths, sewing threads, gas mantles, fishing nets and marine packing[1]. Despite its economic importance, the molecular genetics of ramie and its genomic structure have not been studied until Jie et al.'s report on the genetic relationship of ramie cultivars analyzed with RAPD markers[2].

Microsatellites, or simple sequence repeats (SSR) have been proved to be highly informative DNA markers for their high degree of polymorphism, codominant mode of inheritance and simple data interpretation[3]. They have been successfully used for fingerprinting[4], genome mapping[5], phylogenetic and genetic relationship studies[6], and marker assisted breeding[7]. The advantages and wide uses of microsatellites have promoted the microsatellite development in different plant species such as rice[8], pine[9], white spruce[10], sunflower[11] and others.

However, SSR assay requires the knowledge of genomic sequence to design specific primers, and the development of SSR marker is time-consuming and expensive. Then, an alternative method to SSR, called inter-simple sequence repeats (ISSR) has been described[12]. ISSR employs anchored SSR as primers to amplify a DNA sequence delimited by two inverted microsatellites. Such amplification does not require genomic sequence information and leads to multilocus and highly polymorphous patterns[12]. ISSR fingerprinting has been successful in many crops such as maize[13], rice[14] and flax[15].

In this paper, we report the development of thirteen microsatellite markers in ramie and their polymorphism in ramie cultivars. Further, we

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compare SSR and ISSR for evaluating the genetic relationship in certain representative cultivars of ramie.

1 Materials and methods

1.1 Plant material and DNA isolation

Nineteen cultivars (Table 1) of ramie, representing different root types and geographically separated locations in China and Indonesia, were selected for this study. Total genomic DNA was isolated from fresh leaf material by the method described by Guo et al.[16].

Table 1. Description of the ramie cultivars used for polymorphism screening

Code	Cultivar	Origin	Root type
1	Tiesima	Hunan, China	Middle
2	Baipidou	Jiangxi, China	Middle
3	Yuanjiangyeqingma	Hunan, China	Middle
4	Pingjiangcongdouma	Hunan, China	Deep
5	Ziyangdayepao	Shanxi, China	Deep
6	Yongshanzhuma	Yunnan, China	Shallow
7	Wanzizhuma	Guizhou, China	Deep
8	Chuanzhu-2	Sichuan, China	Deep
9	Baishaqingma	Guangxi, China	Middle
10	Hongyuanma	Guizhou, China	Middle
11	Guanlingyuanma-2	Guizhou, China	Deep
12	Hongpixiaoma	Sichuan, China	Deep
13	Dayezhi-1	Sichuan, China	Middle
14	Yihanzhuma	Sichuan, China	Middle
15	1504	Hubei, China	Deep
16	Huanganyema	Hubei, China	Deep
17	Nanpinzhuma-1	Yunnan, China	Middle
18	Yinni-1	Indonesia	Middle
19	Jiaoaiqinma	Guangxi, China	Deep

1.2 Construction of the microsatellite-enriched library

The microsatellite-enriched library was constructed according to the method described in Refs. [17, 18] with the following modification. RAPD amplification was carried out using 50 pieces of 10-mer random primers (Sangon). All RAPD-PCR products were mixed, and purified with phenol. An aliquot of mixed RAPD-PCR products was ligated to pMD18-T vector (TaKaRa) using DNA ligation kit (TaKaRa) and the ligation mixture was transformed into competent DH5α cell. The transformed cells were plated on LB solid medium in the presence of isopropyl-beta-D-thiogalactopyranoside (IPTG) and X-gal for selection of the positive colonies.

1.3 Isolation of clones containing microsatellite motifs

The white colonies were inoculated separately in 3 mL of LB medium, and grew at 37 ℃ overnight. The plasmids were extracted from them and the microsatellite-containing clones were screened by PCR isolation of microsatellite arrays method[18]. The microsatellite specific primers used in the screening experiment included YHY $(GA)_{15}$, VRV $(CT)_{15}$, VRV $(TG)_{15}$, HBH $(AG)_7$, VBV $(AT)_7$, and DBD $(AC)_7$.

1.4 Sequencing and primer design

All positive clones were sequenced using the ABI 377 automatic sequencer (Perkin Elmer) to confirm the presence of microsatellite arrays and once confirmed, primers of suitable length and base composition that flanked the locus were designed using a software (Net Premier, Premier BioSoft International).

1.5 SSR-PCR

To determine the polymorphism, the DNA of 19 selected ramie cultivars (Table 1) was amplified using each pair of SSR primers. Each PCR reaction was performed in a total volume of 25 μL using the Mastercycler Gradient (Eppendorf). The reaction mixture contained 50 ng of genomic DNA, 2.0 mmol/L $MgCl_2$, 0.1 mmol/L of each dNTP, 0.2 μmol/L each primer, 1× PCR buffer, and 1 U ExTaq DNA polymerase (TaKaRa). The PCR condition was as follows: a pre-incubation at 94 ℃ for 2 min, followed by 36 cycles of 45 s at 94 ℃, 45 s at the appropriate annealing temperatures (see Table 2) and 1 min at 72 ℃, and a final extension step of 5 min at 72 ℃. The PCR products were fractionated on 6% denaturing polyacrylamide gels containing 7 mol/L urea, then visualized by silver staining and photographed.

1.6 ISSR-PCR

For ISSR-PCR, primers were synthesized based on UBC set (http://www.biotech.ubc.ca/frameset.html) and the sequences suggested by Wiesner et al.[15] (Table 3). Each PCR reaction contained 50 ng of genomic DNA, 2.5 mmol/L $MgCl_2$, 0.1 mmol/L of each dNTP, 0.2 μmol/L primer, 1× PCR buffer (TaKaRa), and 1.5 U ExTaq DNA polymerase (TaKaRa). The PCR condition was as follows: a pre-incubation at 94 ℃ for 2 min, followed by 36 cycles of 45 s at 94 ℃, 45 s at the appropriate annealing temperature (see Table 3)

and 1 min at 72 ℃, and a final extension step of 5 min at 72 ℃. Fragments were separated on 2% agarose gels, stained with ethidium bromide, visualized with ultraviolet light and photographed.

Table 2. Properties of *Bodhmeria nivea* microsatellite loci: primer sequence, optimum annealing temperatures (T_a), and size of alleles

Locus	GenBank Accession No.	Motif	Primer sequences (5′→3′)	Size of allele (bp)	T_a (℃)
BN1	AY266319	$(AT)_3(GC)_2AT(GA)_7$	F: ATAGGAATACCAATCGTCGCTCAG R: TGCCGCCACCATCAACTAAT	254	55
BN 2	AY266320	$(AT)_{10}(AATT)_3$	F: GAACGACTCCATCAATAGG R: GAACGGACTCAAAATGAAAC	171	52
BN 3	AY266321	$(AT)_4(GT)_4$	F: CTCCTTTGACACCTCCTT R: CAACAATCAATCACATCCAC	293	49
BN 4	AY266322	$(T)_4C(T)_{13}C(T)_{10}(TC)_6$	F: ACGTCGGTGAAGACTGGC R: GGCAATCCATGTAGCAGAG	258	54
BN 5	AY266323	$(TA)_7$	F: CCAAAGTGTCGCAGCTC R: ATCTGCCGAACTATGTTTG	275	51
BN 6	AY266324	$(AT)_6(T)_5(GGA)_5$	F: TTGGTGGATGTATTAGCC R: GTCCACACATCCATAGAAG	329	47
BN 7	AY266325	$(TTA)_3(AT)_2CGG(TA)_2$	F: CGTCTGGTTTGCAGGTGA R: CACGCTATCCCTCGCAG	213	55
BN 8	AY266326	$(AT)_4(TTA)_2$	F: AGCAGTGACGGATCTAGG R: GATGAGACCTCGTCGAGT	154	49
BN 9	AY266327	$(GA)_{11}$	F: CTCACCGTCCGCAAATCG R: GTACAACCCTGACCTCCTCAAG	138	58
BN 10	AY266328	$(CT)_8G(TTC)_2(GC)_2$	F: CCATTGATGAAGGACGATAG R: CACTACCACTATGCTACGGAA	236	53
BN 11	AY286048	$(RT)_8(GT)_6(AT)_7$	F: GCTTGGTCTAATCTCTTG R: GCACTTCTATGTGACTTATG	202	44
BN 12	AY286048	$(AT)_8$	F: ACATAAGTCACATAGAAGTGC R: GTCGGCTTCTAACACATC	219	47
BN 13	AY286049	$(AT)_7$	F: CATATCATGAATGGTGTTTGAG R: GTGAATGTGTTATGTTTCAATTAC	156	50

Table 3. Primers used in ISSR assay

Primer	Primer sequences	T_a (℃)
3PCT1	5′-YHY$(GA)_{15}$-3′	65
3PCT2	5′-VRV$(CT)_{15}$-3′	65
3PCT5L	5′-VRV$(TG)_{15}$-3′	60
UBC882	5′-VBV$(AT)_7$-3′	42
UBC884	5′-HBH$(AG)_7$-3′	57
UBC889	5′-DBD$(AC)_7$-3′	57

1.7 Data analysis

The degree of polymorphism was measured by evaluating the differences in alleles/bands number per assay, polymorphism information content (PIC), and number of phenotypes detected per assay. The PIC was calculated according to Wiesner et al.[15]: $PIC_j = n(1-\sum P_{ij}^2)/(n-1)$, where n is the sample size, and P_{ij} the frequency of the i-th pattern revealed by microsatellite primer j summed across all patterns revealed by the primer j.

Electrophoretic data was collected as the method described by Paniogo et al.[11], presence or absence of each band was coded as 1 and 0, respectively, in a binary matrix. The cluster analysis of electrophoretic data was calculated by SPSS 11.0.1 software package (SPSS Inc, USA), from which a variant of pearson correlation coefficient and the average linkage (between the groups) clustering procedure were selected. The relationship of ramie cultivars was represented graphically in the form of dendrogram.

2 Results

2.1 Microsatellite-enriched library construction and microsatellite screening

A total of 50 pieces of 10-mer random primers were used to amplify ramie DNA. An aliquot of mixed amplified products was cloned into pMD18-T vector. A total of 250 insert-bearing clones from the library were screened by PIMA method[18], and 26

clones were identified as positives. We sequenced these positive clones and found that only 12 clones contained a microsatellite motif, and one of these clones contained two microsatellite loci (BN11 and BN12). Table 2 shows the sequences and optimal annealing temperature of the 13 primer pairs that were derived from the sequence of 12 clones containing microsatellite motifs. Of these 13 loci, 4 loci (BN5, BN9, BN12 and BN13) were complete di-nucleotide repeat locus, others contained a complex SSR with a mixed repeat motif. In addition to di-nucleotide locus, three of these clones contained a tri-nucleotide repeat (BN6, BN7, BN10) and one clone contained a tetra-nucleotide repeat (BN2).

2.2 Polymorphism of SSR

The 13 primer pairs flanking the microsatellite motifs were used to amplify the DNA from 19 selected ramie cultivars, one of amplification patterns is shown in Fig. 1. Table 4 summarizes the number of alleles, number of polymorphic alleles and polymorphic index content (PIC). The number of alleles ranged from 2 to 5, with average of 3.15 per locus. The 13 loci produced a total 41 alleles, of which 39 (95.1%) were polymorphic in the 19 ramie cultivars. The difference of polymorphism among the 13 microsatellite loci is further reflected in the PIC. The PIC ranged from 0.199 (BN12) to 0.713 (BN2), the average PIC is 0.441. As could be seen from Table 2 and Table 4, there appears to be no correlation between polymorphism (polymorphic bands and PIC) and motif of microsatellite locus.

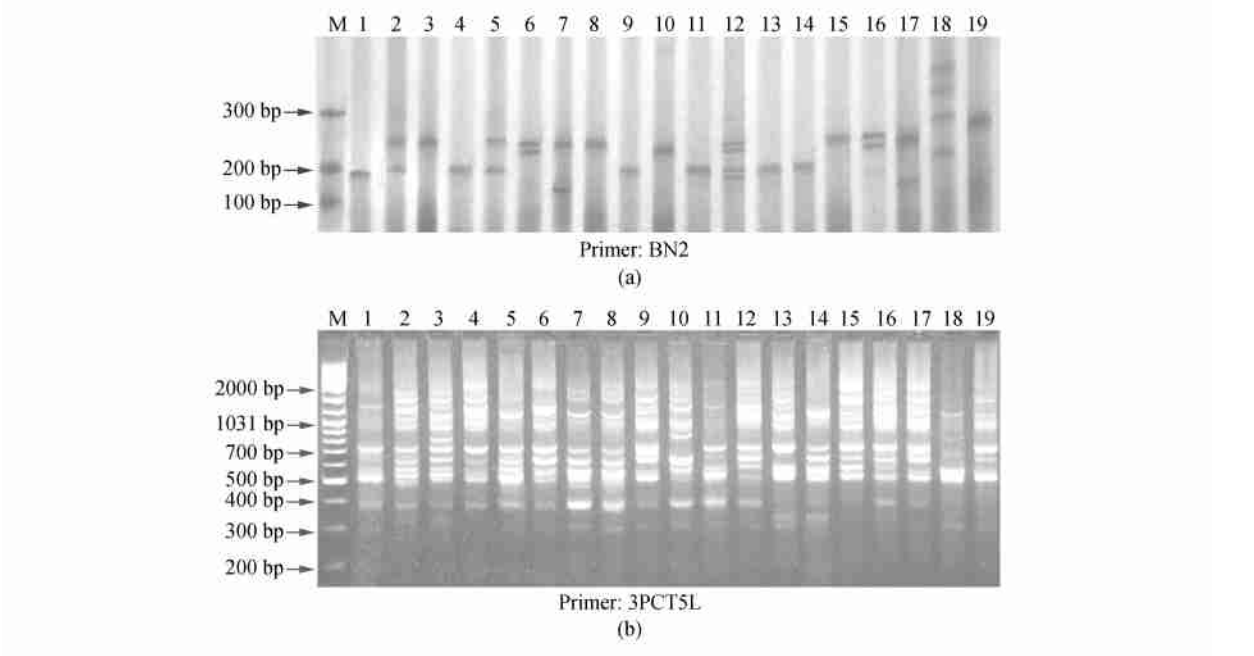

Fig. 1. Polymorphisms detected in 19 selected ramie cultivars using SSR (a) and ISSR (b). M, DNA marker; 1—19 represent 19 cultivars listed in Table 1.

2.3 Polymorphism of ISSR

A total of 6 pieces of ISSR primers were used for the genetic analysis of the 19 ramie cultivars. All primers were able to produce clear and reproducible band pattern except UBC882 (Table 4), one of amplification patterns is shown in Fig. 1. The number of bands ranged from 7 (3PCT1) to 12(UBC 889), with average of 9.4 bands per primer. The five primers (3PCT1, 3PCT2, 3PCT5L, UBC 884 and UBC 889) produced a total of 47 bands, of which 34 bands (72.3%) were polymorphic. The primer UBC 882 gave no scorable band at all, possibly because the AT repeat motifs formed into dimmer in PCR amplification. The PIC ranged from 0.578 (3PCT1) to 0.744 (3PCT5L).

2.4 Relationship analysis of ramie cultivars

The SSR and ISSR amplification patterns were used to determine the genetic similarity matrices, which were then used to construct dendrograms. Results from the cluster analysis based on microsatellite data are given in Fig. 2. The cultivars with the same root type and from the near origin were clustered into one group. The results proved that the

SSR markers could be used in relationship analysis of ramie cultivars.

Table 4. A summary of banding patterns generated by SSR and ISSR assay

Locus or primer	Number of bands	Number of polymorphic bands (%)	PIC
SSR locus			
BN1	3	3 (100)	0.503
BN2	5	5 (100)	0.713
BN3	3	3 (100)	0.556
BN4	4	3 (75.0)	0.614
BN5	3	3 (100)	0.292
BN6	3	3 (100)	0.292
BN7	3	3 (100)	0.515
BN8	3	3 (100)	0.585
BN9	5	4 (80.0)	0.380
BN10	2	2 (100)	0.409
BN11	3	3 (100)	0.380
BN12	2	2 (100)	0.199
BN13	2	2 (100)	0.305
Average	3.15	3.00 (95.1)	0.441
ISSR primer[a)]			
3PCT1	7	5 (71.4)	0.578
3PCT2	8	6 (75.0)	0.666
3PCT5L	9	7 (77.8)	0.744
UBC884	11	8 (72.7)	0.656
UBC889	12	8 (66.7)	0.612
Average	9.4	6.8 (72.4)	0.651

a) Primer UBC882 gave no scorable bands.

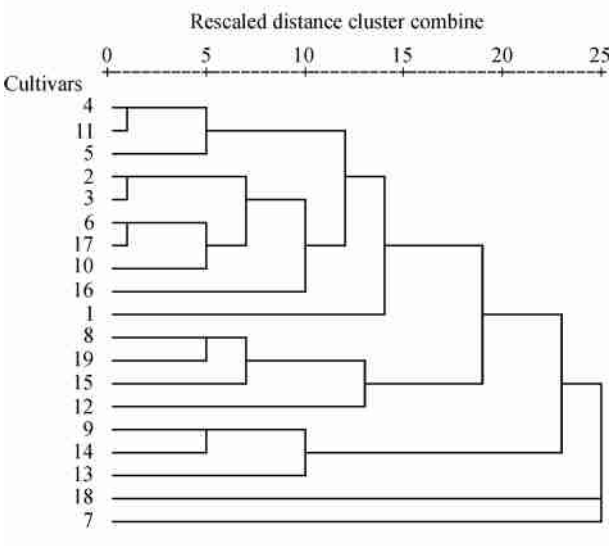

Fig. 2. Dendrogram of 19 ramie cultivars established by using SSR markers.

3 Discussion

RAPD is known as a method to enrich microsatellite[17]. In our case, we selected 50 pieces of 10-mer random primers to amplify the ramie DNA, which produced large numbers of different bands, and these fragments were ligated to the pMD18-T vector, and a library was constructed. A total of 250 insert-bearing clones from the library were screened by PIMA method[18] and the positive clones were sequenced. Twelve clones (5.2%) containing microsatellite motif were identified. Compared with traditional isolation method, by which only 0.4% to 3.1% positive clones can be obtained[19], PIMA method has many advantages: it costs less, is easy to use and avoids using radioactive materials, and with a high efficiency.

In our experiment, both SSR and ISSR markers were able to uniquely fingerprint each of the 19 ramie cultivars. However, they differed in the number of bands scored and the polymorphism detected. For example, the number of bands scored for SSR was 3.15 bands per locus, while it was 9.40 bands per primer for ISSR. The level of polymorphism detected by SSR was 95.1%, higher than that by ISSR (72.3%). Similar results were observed when Nagaraju et al.[20] compared SSR with ISSR in silkworm where the level of polymorphism was 86% with SSR compared to 76.98% with ISSR. Although ISSR produced a lower level of polymorphism compared to SSR, the average PIC of ISSR (0.651) was higher than that of SSR (0.441) in our study, and PIC directly corresponds to the power of molecular marker to discriminate cultivars[15]. The higher PIC value of ISSR reflects that ISSR is more efficient for fingerprinting ramie cultivars than SSR. However, ISSR loci are dominant, rather than codominant, which cannot reflect the allelic status of polymorphic bands, and hence homozygosity levels cannot be determined in population studies[20], while SSR loci are codominant, and they can be used in determining the homozygosity levels, this property is very important for genetic analysis of ramie, because ramie is a cross-pollinated perennial herb, its genetic background is complicated, and especially its homozygosity level is difficult to determine. Moreover, SSR loci are inherited in a codominant Mendelian manner, the polymorphic SSR loci can be mapped to the genome using a segregating population[21,22]. So, SSR is more suitable for the construction of linkage map, quantitative trait loci (QTL) studies of complex traits and marker-assisted selection. Additionally, SSR-PCR pattern is more reproducible and clearer than ISSR-PCR pattern, because SSR primers come from the conserved regions flanking the loci. Although the

isolation and characterization of SSR is time-consuming and expensive, they will provide more information on genetic data in a very short period of time.

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