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Molecular identity of ramie germplasms using simple sequence repeat markers

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ABSTRACT. DNA identity is highly effective and efficient for distinguishing crop varieties regardless of their phenotypic similarities. To establish DNA identity in ramie, 21 simple sequence repeat primers were amplified in 108 accessions of domestic and exotic ramie germplasms. Sixty polymorphic bands were obtained, with an average of 2.9 bands per locus and 2-8 band types per primer locus (average of 5.19 band types). The Simpson's diversity index of the 21 simple sequence repeat loci ranged from 0.158 to 0.808 with an average of 0.612. There was large difference in the specific index in the germplasm tested, from 44.082 to 218.163, with an average of 83.620. Based on allele band type, 8 primer pairs were selected for DNA fingerprinting of the 108 genotypes. The combination of the 8 primer pairs were found to be very effective for distinguishing these genotypes, indicating that they can be used in the molecular DNA identity of ramie.

Key words: Molecular identity; Ramie; Simple sequence repeat

INTRODUCTION

Ramie (*Boehmeria nivea* L.), also called Chinese grass, is principally used for fabric production and is one of the oldest fiber crops cultivated in China; this crop has been cultivated for more than 6000 years. It is a popular perennial plant and native to eastern Asia (Xiong, 2008). The bark of the vegetative stalks is the source of bast fiber. A series of ramie industry products have become popular in domestic markets, including shirts, underwear, and socks (Liu, 2002). In previous studies of ramie, various applications of ramie were established, with the exception of the fiber industry. Ramie is also cultivated as fodder crop in the southern regions of China because of its excellent nutritional value, containing 22% crude protein, 18% crude fiber, 1% Lys, 4% Ca^{2+}, and other nutrient elements needed by livestock regardless of the fresh leaf or its processing into dry power or silage (Yu et al., 2007; Xiong et al., 2010). Recently, ramie has become an optimal crop for conserving soil and water in the Yangtze River valley because of its abundant roots and luxuriant foliage (Tu and Chen, 2007).

In the ramie germplasm collection process, it is necessary to conduct cultivar identification for breeding selection and germplasm reservation. Traditionally, ramie germplasms are identified based on morphology, but this is laborious. Morphological traits must be obtained through multi-years and multi-sites, which is time consuming and costly. Additionally, the differences between morphological traits among germplasms are sometimes difficult to identify.

Molecular markers have been widely used to characterize ramie germplasms. For instance, random amplified polymorphic DNA (RAPD) (Jie et al., 1999; Guo et al., 2001; Meng et al., 2010), simple sequence repeats (SSR) (Zhou et al., 2004; Zou et al., 2012; Guo et al., 2013), inter-simple sequence repeat (ISSR) (Liu et al., 2006; Hou et al., 2006; Ding et al., 2008), sequence-related amplified polymorphism (SRAP) (Liu et al., 2008; Wen et al., 2011; Zou et al., 2012), restriction site amplification polymorphism (Zou et al., 2012), and random amplified microsatellite polymorphism (Zhou et al., 2004) were developed and shown to be rapid, accurate, and economical in ramie germplasm research. Moreover, several reports have illustrated the advantage of using molecular ID in crop species. Zheng et al. (2010) established 51 kenaf germplasms using of ISSR and RAPD markers. Liu et al. (2013) established 127 kenaf germplasms using SRAP markers. Wang et al. (2010) first distinguished 42 germplasms of ramie using 7 ISSR markers, and developed the first molecular ID of ramie. However, this plant shows poor stability and it is complex to record molecular ID because of the 16-digit code in the ID constructed using ISSR markers. Comparing with ISSR and other molecular markers, SSRs are highly polymorphic, informative, codominant, technically simple, and reproducible, and have become commonly used for constructing the molecular ID in the crop species (Fang et al., 2001). Some species have been evaluated by molecular ID using SSR markers. Gao et al. (2009) established 83 soybean germplasm molecular IDs using 9 SSR markers. Yang et al. (2010) established 36 tea germplasm molecular IDs using 17 SSR markers. Chen et al. (2011) established 202 peach germplasm molecular IDs using 8 SSR markers. Wang et al. (2011) established 142 sweet sorghum variety molecular IDs using 11 SSR markers. Zhang et al. (2014) established 20 pear variety molecular IDs using 2 SSR markers. However, there have been no reports related to molecular ID using SSR markers in ramie.

Therefore, the objective of this study was to construct molecular IDs of the 108 ramie germplasms using SSR markers.

MATERIAL AND METHODS

Materials and DNA isolation

A total of 108 ramie accessions (Table 1) growing in a national ramie germplasm nursery affiliated with the Institute of Bast Fiber Crops, CAAS, China, were used in this study. DNA was isolated from young leaves collected from each ramie accession using the DNeasy plant mini prep kit (Qiagen, Hilden, Germany).

Table 1. A hundred eight germplasms in the study.

Code	Entry	Origin	Code	Entry	Origin
1	Wayaozhuma	Guangxi	55	Xinminqingma	Guizhou
2	Ganzaerhao	Jiangxi	56	Youqima	Hunan
3	Miaobazhuma	Sichuan	57	Baiyema	Jiangxi
4	Simaohongzhuma	Yunnan	58	Lichuanhoupizhuma	Jiangxi
5	Shuiqingzhuma	Chongqing	59	Boyanghuangyema	Jiangxi
6	Yichuntongpiqing	Jiangxi	60	Xiaoqinggan	Sichuan
7	Yachibaima	Guizhou	61	Yihuangjiama	Jiangxi
8	Rongchangzhuma	Chongqing	62	Xiangningdayelv	Hubei
9	Guangdonghuangpidouerhao	Guangdong	63	Yedouzi	Jiangxi
10	Qingyezhuma		64	Tiantaitiema	Zhejiang
11	Hexianjiama	Guangxi	65	Dayehongzhameng	Jiangxi
12	Guangpimayihao	Jiangxi	66	Jiangkouqingpizhuma	Guizhou
13	Juandongtuma	Chongqing	67	Longtangbaima	Chongqing
14	Limuqingma	Guizhou	68	Wuchangshanpozhumayihao	Hubei
15	Dadaoma	Guizhou	69	Jinpingqingma	Guizhou
16	Lipingqingma	Guizhou	70	Qingpigan	Jiangxi
17	Lidazhuma	Yunnan	71	Fenyihuangguangdou	Jiangxi
18	Gebuqingma	Guangxi	72	Huangjiuma	Hunan
19	Tongmuqingma	Guangxi	73	Guangpimaerhao	Jiangxi
20	Qingpidamayihao	Chongqing	74	Qianzhuyihao	Guizhou
21	Gaoanma	Jiangxi	75	Loushanhuangpima	Guizhou
22	Yujiangma	Jiangxi	76	Anlongzhumaerhao	Sichuan
23	Fulisima	Chongqing	77	Linggongqingpima	Guizhou
24	Wuchuanbaima	Guizhou	78	Japanzhumaqihao	Japan
25	Huanhancongma	Sichuan	79	Xieliqingma	Chongqing
26	Nanchongzhuma	Sichuan	80	Kuguaqing	Hunan
27	Xiningxianma	Chongqing	81	Longhuibaimayihao	Hunan
28	Puqidayelvyihao	Hubei	82	Yongshanzhuma	Yunnan
29	Wulonghonggan	Chongqing	83	Honggujin	Hubei
30	Xiaogubai	Jiangxi	84	Yushanma	Jiangxi
31	Rongjiangbaimayihao	Guizhou	85	Yinniyihao	Indonesian
32	Niutima	Hunan	86	Sichuangaodibaima	Chongqing
33	Nanchenghoupizhuma	Jiangxi	87	Yinnierhao	Indonesian
34	Huangqingdou	Jiangxi	88	Shanqingbaima	Chongqing
35	Zixima	Jiangxi	89	Ershiqingmaerhao	Hubei
36	Datianhuangganzhuma	Yunnan	90	Xinningqingma	Hunan
37	Ningduyema	Jiangxi	91	Ximatuma	Guangxi
38	Heipima	Guangxi	92	Yinnisanhao	Indonesian
39	Tianbaomayihao	Jiangxi	93	Zunyichuangenma	Guizhou
40	Changshaqingyema	Hunan	94	Dayujiandaobai	Hubei
41	Chuanzhuerhao	Sichuan	95	Xiaoyelugan	Jiangxi
42	Nanchengbaopizhuma	Jiangxi	96	Pingchangjiama	Sichuan
43	Ningduqingzhuma	Jiangxi	97	Leiyanghuangkema	Hunan
44	Manqiangzhuma	Hubei	98	Gaodiqingma	Chongqing
45	Lvzhubai	Jiangxi	99	Dingyezhuma	Guangxi
46	Dayujiandaobai	Hubei	100	Kanlazhuma	Myanmar
47	Quxianzhuma	Zhejiang	101	Shengbaxianma	Hubei
48	Zhuzibian	Jiangxi	102	Xinpuqingma	Guizhou
49	Yangshuojigubai	Guangxi	103	Tianpaishanyema	Guangxi
50	Hupima	Jiangxi	104	Huangjindou	Hubei
51	Yangxinxiyelv	Hubei	105	Chongrenzhuma	Hunan
52	Xiangzhuliuhao	Hunan	106	Gegenma	Hunan
53	Ningdudabaima	Jiangxi	107	Tianbaomaerhao	Jiangxi
54	Xiangzhuyihao	Hunan	108	Huangpinghuangganma	Guizhou

SSR primers

Twenty-one SSR primer combinations (Table 2) were synthesized according to Chen et al. (2011).

Table 2. Primer sequence of simple sequence repeat (SSR) used among ramie accessions.

Locus	Primer sequences (5'-3')	Primer sequences (5'-3')
b50	F: AACAATCCAGGAGTGGCAATC	R: ACAAGCGAAGATCGTCTCATC
b35	F: CGTTCAGTCACCAGCAAGG	R: GAGGGAAGCAGGGAGAGC
b38	F: TAATCCCTCAATGGCTCTTTTC	R: GAGAAGGATACGAATTGACAGG
b40	F: TGTATAGAACTGAGTAAATGATTG	R: CAACTTTCTTAAACCACTTTCG
b43	F: CGAGCCTTCTTCTTCTTCTGG	R: GCAAGCAATACGGACAGTAGG
c03	F: CGTGAAAATAGTGATATGTGTG	R: ACTGTAACAATCAAGAAGAAACC
c07	F: GCCACAGCCGAGGAAGAG	R: TCTCATCACCACCACCTTAGG
b27	F: AGCCAGGTTCCAGAAGTCC	R: CATAATCACAAAGTCTCGGTTCC
b28	F: TCCCACCACGGACTACTG	R: AACCACCATCATCATCATCATC
b11	F: GCGGAGGCTTAATTTGCTTTG	R: ACTCAATACATACACGGCACTAG
b16	F: ACCTCTACGGACCTCTTCTTC	R: CATAACATAACATGACACACAAGC
b24	F: GAGCCAGAGCCAGGTTCC	R: ACAAAGTCTCGGTTCCTTACAC
b34	F: AATAGAATGTGGAGGCGATAGAG	R: AAACCATAAATCAACTACCGAACC
b64	F: CTTGAGATACAGCCTTCCATTAG	R: CACACCTCGCTTCCCTTG
c17	F:GAAACTATTTCCACCAACAAAG	R: ACACACATTCCTACACACC
b57	F: CGGATATGGTGGAGGTTATGC	R: CAGAACGACGACGACGAC
b65	F: ACGAACCACAACACAGAGAG	R: ACGAGGGAACACCAGAGAG
c18	F: AAGCCGAGCGTGAAGAAG	R: ACACACAGAAAGAACACAAGAC
b53	F: GGCTCAAGTTTGCTCATAGATTC	R: CGGCTTCGCTTTAGGATTTG
b56	F: CGGTCTGTGGATACGAATGG	R: GACGACGACGACGATGATG
c10	F: AGTGCGGAGATAACTGTTC	R: GGCTACTTTATTCTAAACCAAAC

SSR analysis

SSR-polymerase chain reactions (PCRs) were carried out in 10-μL reaction volumes with 1X PCR buffer, 0.2 mM dNTPs, 1 U *Taq* DNA polymerase (Tiangen, Beijing, China), 0.5 μL forward primer (10 nM, Tiangen), 0.5 μL reverse primer (10 nm, Tiangen), and 0.5 μL DNA of each accession under the following PCR conditions: 5 min at 94°C, followed by 30 cycles for 30 s at 95°C, 30 s at the primer-specific annealing temperature, 30 s at 72°C, and final extension for 10 min at 72°C.

PCR products were separated on 8% polyacrylamide gels, and silver staining was conducted according to the method described by Zhang et al. (2000). Molecular weights were estimated using a DNA marker (DNA Marker 2000, BioTeke Co., Beijing, China). SSR analysis was repeated at least twice. Clear bands from PCR products were recorded types 1-8. Faint bands were recorded as 0.

Diversity index (DI) of SSR primers and CHEN specialty index (CHEN-SI) of germplasms

The diversity index of SSR primers and CHEN specialty index (CHEN-SI) of germplasms were calculated using Genetics Statistics 3.0 (2007SR11872) developed by North-East Agriculture University, China.

Simpson's DI (Simpson):

$$D = 1 - \sum(Pi^2) \quad \text{(Equation 1)}$$

In the formula, Pi was the proportion of band types of a locus.

Simpson's unbiased DI (unbiased Simpson):

$$D = 1 - \sum((ni \times (ni - 1)/(N \times (N - 1))) \quad \text{(Equation 2)}$$

In the formula, ni was the number of band types in a locus; N was the total number of all band types counted in a locus.

Shannon-Weiner's DI (Shannon-Weiner):

$$H' = -\sum(Pi \times \log(Pi)) \quad \text{(Equation 3)}$$

In the formula, Pi was the proportion of band types in a locus.

Brillouin's DI (Brillouin):

$$H = (1/N) \times \log(N!/n1!n2!n3!...ns!) \quad \text{(Equation 4)}$$

In the formula, n1!n2!n3!...ns! was the factorial product of each band type in a locus.

CHEN specialty index (CHEN-SI):

$$\text{CHEN-SI}i = (\sum(1/(Nij/Mj)))/(m \times n) \ (i = 1,2,3...m; j = 1,2,3...n) \quad \text{(Equation 5)}$$

In the formula, Nij was the total distributive number of band type in the m individual of the i^{th} individual in the j locus; Mj is the total number of band type of the j locus, its number is m in the data of dominant molecular marker.

The Chi-square analysis was used to analyze the data. $P \leq 0.01$ indicated extreme significance; $P \leq 0.05$ indicated significance; and $P > 0.05$ indicated no significance.

ID construction

The molecular ID of ramie germplasms was constructed using ID Analysis 3.0 (Northeast Agriculture University, China).

RESULTS

Band type developed by SSR primers

The number of alleles per locus ranged from 2 to 3 among the 21 primer pairs. Fourteen primers amplified 2 alleles, while 7 primers amplified 3 alleles.

The number of band types developed by SSR primers was 2-9. Of the 21 primers, b50 amplified the largest number of band types (9 band types), while b64 and c17 amplified the smallest number of band types (2 band types).

DI of SSR primers

The DI of SSR primers is listed in Table 3, including 4 type DI consisting of Simpson's diversity index, Simpson's unbiased diversity index, Shannon-Weiner's diversity index,

and Brillouin's diversity index. Values ranged from 0.158 to 0.808, 0.159 to 0.816, 0.146 to 0.747, and 0.133 to 0.702, respectively. Among the 21 SSR primers, primer c10 showed the highest DI and primer c07 showed the lowest DI.

Table 3. Diversity of simple sequence repeat (SSR) primers.

SSR primers	Band type	Simpson	US	SW	BR
b50	9	0.751	0.758	0.663	0.619
b35	5	0.701	0.708	0.55	0.521
b38	4	0.527	0.532	0.349	0.332
b40	7	0.664	0.67	0.569	0.532
b43	3	0.477	0.481	0.291	0.281
c03	5	0.688	0.694	0.542	0.514
c07	4	0.158	0.159	0.146	0.133
b27	4	0.614	0.62	0.44	0.421
b28	6	0.619	0.626	0.487	0.455
b11	4	0.664	0.67	0.475	0.456
b16	4	0.602	0.608	0.428	0.408
b24	4	0.641	0.647	0.46	0.44
b34	5	0.587	0.592	0.455	0.43
b64	2	0.356	0.359	0.235	0.225
c17	2	0.486	0.491	0.295	0.285
b57	4	0.672	0.678	0.543	0.516
b65	7	0.617	0.623	0.522	0.488
c18	8	0.735	0.743	0.701	0.65
b53	7	0.709	0.715	0.636	0.595
b56	8	0.783	0.791	0.715	0.666
c10	7	0.808	0.816	0.747	0.702

Simpson indicated Simpson's diversity index; US indicated Simpson's unbiased diversity index; SW indicated Shannon-Weiner's diversity index; BR indicated Brillouin's diversity index.

Special band type

Two primers amplified special band types. The 5th special band type developed by primer b50 could distinguish Lipingqinma among 108 ramie germplasms. The 1st and 5th special band types developed by primer b65 could distinguish Ganzaerhao and Shanqingbaima, respectively.

CHEN-SI of germplasm

The CHEN-SI values of 108 germplasms are listed in Table 4. There were significant differences ($P < 0.01$) among the CHEN-SI of the 108 ramie germplasms, ranging from 44.082 to 218.163. There were 2 germplasms with CHEN-SI values greater than 200, including the 88th with 218.163 and the 16th with 201.988. There were 4 germplasms showing CHEN-SI values less than 50, including the 68th with 49.825, the 69th with 48.216, the 70th with 45.86, and the 74th with 44.082. The high CHEN-SI indicates that ramie germplasms have a large number of special band types, and thus the results may be useful for identifying and preserving germplasm resources of ramie.

Germplasm ID construction

The PCR amplification banding pattern of 108 ramie germplasms was recorded, which was amplified by 21 SSR primer combinations, as well as being expressed 1, 2…n, respectively. Next, the molecular identity card of ramie was constructed according to the following

Table 4. CHEN index and ID of germplasms.

Germplasm code	CHEN index	ID	Germplasm code	CHEN index	ID
1	79.267	43311132	55	99.914	17646213
2	176.671	23122223	56	95.824	12326313
3	74.214	23413323	57	69.994	74323113
4	52.968	24434323	58	153.699	35326332
5	71.503	12435223	59	101.934	47640132
6	56.267	24221233	60	136.545	11324132
7	58.93	54326123	61	67.944	43325322
8	96.198	25221113	62	97.201	74626233
9	92.772	42444113	63	59.451	44235332
10	85.013	45425323	64	82.951	32211132
11	98.284	37214303	65	98.17	34415312
12	92.397	54215323	66	95.376	30265122
13	107.657	27221113	67	59.35	30244122
14	73.91	24336333	68	49.825	74236133
15	98.006	32227223	69	48.216	50236133
16	201.988	32453122	70	45.86	44224123
17	68.989	34436223	71	75.213	34231122
18	79.796	25248123	72	80.116	17221222
19	72.202	24223213	73	96.707	14446232
20	81.511	24221123	74	44.082	40256033
21	57.924	22334133	75	61.489	54233133
22	98.259	24321233	76	59.744	44233032
23	87.989	13224123	77	68.141	25221133
24	89.338	55201333	78	72.132	54220233
25	59.163	34323323	79	89.832	72334332
26	80.615	35329322	80	81.766	44424122
27	92.963	45221232	81	96.743	4465123
28	85.17	24625223	82	85.892	77333223
29	123.05	37225222	83	85.563	37621333
30	71.814	54221133	84	57.149	44225132
31	68.646	43226223	85	59.287	20225132
32	57.626	34233323	86	110.208	72334222
33	59.482	54221333	87	55.507	2225232
34	68.98	27226323	88	218.163	21541132
35	76.586	53225323	89	89.272	23424323
36	130.457	27235323	90	58.964	54224222
37	58.597	7226123	91	84.068	72430222
38	62.598	37234123	92	66.301	72237322
39	110.406	54215123	93	96.842	27637332
40	81.42	43224332	94	84.532	54615322
41	108.933	24265323	95	73.37	77225223
42	70.83	72225113	96	68.476	34225212
43	71.802	52234112	97	61.951	24225232
44	67.845	32249322	98	74.089	14243222
45	96.457	44424323	99	67.123	22234132
46	97.761	34231123	100	62.668	47321322
47	84.048	34224223	101	80.758	25313123
48	75.369	44214103	102	67.52	74325232
49	83.857	23217312	103	71.901	14234233
50	69.219	24221312	104	62.803	54334132
51	74.177	44426312	105	69.151	34432323
52	108.04	23418313	106	82.076	24325222
53	66.518	24231213	107	81.69	22333332
54	95.737	14246213	108	157.241	57224333

steps. First, we deleted SSR primers when the proportion of blurred amplification belt was above 4%. Six SSR primer combinations were deleted, including b35, c03, b28, b16, c18, and b56. The second step was to delete SSR primers when the similarity coefficient was greater than 0.8 with others. Two SSR primers were deleted, including c07 and b24. Finally, we constructed a molecular identity card based on the specificity index of SSR primers. The germ-

plasm resources of ramie were distinguished using the specific allele of more than 2 marker combinations. The distinction would be made by increasing the number of markers until they were not distinguished entirely using 2 markers, i.e. the k-th marker combinations amplified n banding patterns corresponding to the k-th bits of molecular identity. In current study, the 108 germplasm resources of ramie were distinguished using 8 SSR primer combinations (k = 8), including c10, b53, b65, b40, b50, b11, b27, and c17; the 8-bit molecular identity card (ID) was constructed (Table 4). For instance, the molecular ID of ramie known as Wayaozhuma was 43311132. The 1st 4 indicated that the b20 primer (ki) amplified its 4th banding pattern (nj), and the 3rd 3 indicated that the b65 primer amplified its 3rd banding pattern.

DISCUSSION

The banana fingerprints developed by Wang et al. (2009) can be considered molecular identification, and the authors combined letters with numbers to determine the digits of molecular identification and different alleles amplified by SSR primers. For example, the 11th allele amplified by the first marked primers was referred to as A11, resulting in a large number of string code digits in the molecular identification. Wang et al. (2010) previously constructed ramie molecular identification using 0 or 1 notation including the 16-digit code using ISSR markers. In the present study, the amplification results of the Nth marker's primers directly corresponded to the Nth bit in molecular identification, omitting the expression of a tag name. Additionally, the molecular identification only had 1 digit in each position because the amplified band pattern of each labeled primers did not exceed 9. Compared to the previous ramie molecular identification studies, this method is very simple to record.

With the continuous development of DNA molecular markers and improvement of detection technology, identifying crop variety resources traditionally based on morphological characteristics has become focused on the DNA level. Recently, molecular identity cards have been used for variety characteristic digitization. In this study, we concluded that a string code could be applied to express the molecular identification of crop variety resources and to determine the differences among varieties.

Although appraisal using molecular markers has advantages that morphological appraisal does not, there were some limitations to our study. Choosing concrete molecular markers may influence the analysis because of their respective shortcomings. Our previous study established 42 molecular IDs of ramie using ISSR molecular markers. However, ISSR technology is prone to error and deviations because of the large number of amplified bands and the difficulties in duplication. SSR molecular markers frequently result in high duplication. However, this method is easy and does not require a DNA digestion step. These characteristics make it possible to establish molecular IDs. SSR markers are stable over different generations, according to SSR analysis of parents and their descendants in the study of barley genealogy. The SSR stability of different individuals of the same species has been verified in rice (Akagi et al., 1997). Therefore, SSR marker-based molecular ID is more accurate and reliable compared with ISSR markers.

Although we identified 108 ramie germplasms using 8 pairs of SSR primers in this study, the character differences for each germplasm could not be directly determined because the corresponding relationship between various allele and their corresponding agronomic characters were not identified. Although traditional morphological identification requires a longer period and is greatly affected by environmental factors, it is simple, visible, and easy

to operate, which is in contrast to molecular markers. Therefore, this method is applicable for identifying ramie germplasm resources, combining traditional morphological identification with molecular marker identification to establish a system for germplasms ID.

Understanding the genetic background of germplasms is vital for breeding. The samples tested in this study were collected from different origins, indicating the wide applicability for breeding. Thus, the molecular identification (ID) of each species can represent specificity. The results showed that molecular ID is a powerful approach for distinguishing germplasm resources regardless of their origin, genetic pedigree, or morphological similarity. For example, the xiangzhu1 and qianzhu1 ramie varieties have similar genetic backgrounds because they have the same same parent, huangkezao, with the molecular IDs 14246213 and 40256033, respectively. Therefore, molecular ID may provide a reference for germplasm identification and variety selection.

For additional ramie germplasms, a larger number of special bands from special germplasms developed using suitable primers can be used to construct the molecular ID in ramie. However, it is possible for ramie germplasms of different varieties to have the same molecular ID because of the limited number of SSR primers available. Thus, it is necessary to expand the number of SSR primers.

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